ISOLATION AND CHARACTERIZATION OF A β-TUBULIN GENE FROM THE FILARIAL WORM *Onchocerca gibsoni*

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DECLARATION

I hereby declare that I alone have composed this thesis, and that, except where stated otherwise, the work presented within is my own.

Maria Elizabeth Bernardes Margutti Pinto
1990

A meus pais, Pedro (*in memoriam*) e M. Isabel Bernardes, cuja convivência e exemplo de vida me permitiram crer, buscar e alcançar.

Ao Paulo e nossos filhos, Vivian, Leonardo e Claudia, agradeço por todo apoio, paciência, compreensão e carinho no decorrer desse trabalho.

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ABSTRACT

Onchocerciasis or river blindness is a major cause of infectious blindness in the world. In severely affected areas, half of the adult population may be blinded by the disease. The human parasite, *O. volvulus*, is very closely related to the cattle parasite *Onchocerca gibsoni* which provides an abundant source of biological material for analysis. Thus, *O. gibsoni* offers a model system for biochemical analysis and drug screening.

In these organisms, as in other eukaryotes, microtubules are essential, multifunctional, subcellular components. They are involved in chromosome segregation, cell architecture, motility, intracellular transport, and secretion. The major protein of microtubules is tubulin, a heterodimer of two distinct polypeptides designated α and ß. Each subunit has a molecular weight of about 50kD and is encoded by a distinct set of genes. Tubulins are highly conserved proteins and the number of genes coding for both α and ß-tubulin vary dramatically between different species. This major structural protein of eukaryotic cells has particular importance in helminthic parasites as it is a target for anthelminthic benzimidazoles, which directly bind to tubulin and inhibit the assembly of microtubules.

The effectiveness of such drugs depends on differences in the structure of the tubulin molecules from the parasite and its host. In this context, a genomic library from *O. gibsoni* has been constructed, screened with a ß-tubulin gene from *Plasmodium falciparum* and the gene isolated. The chromosomal copy of the gene has been completely cloned and sequenced. This has revealed the gene to possess a very complex structure compared to the organisation of all the known ß-tubulin genes as the coding region is interrupted by 11 introns. The deduced

polypeptide is 444 amino acids long, and its sequence is highly conserved. The position of some introns appear to demarcate functional domains within the protein.

The results that have emerged from this thesis are a step forward not only in the rational design of drugs, but also offer an insight into the biology and evolution of an *Onchocerca* gene and its product.

k	kilo (x 1000)
kb	kilobase
kd	kilodalton
klenow	large fragment of DNA polymerase
K_M	Michaelis Constant
<	less than
min(s)	minute(s)
M	molar
mA	milliampere(s)
mCi	millicurie(s)
mg	milligram(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
mmol	millimole(s)
mRNA	messenger ribonucleicacid
mwt	molecular weight
NAD	ß-nicotinamide-adeninedinucleotide
ng	nanogram(s)
nm	nanometer(s)
nmol	nanomole(s)
OD	absorbance
^{32}P	ß emitting isotope of phosphorus
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
%	percentage
pg	picogram(s)
pH	$-\log^{10}$ (hydrogen ion concentration)
pI	isoelectricpoint
PRPP	5'-phosphoribosyl-1-pyrophosphate
RNA	ribonucleicacid
RNase	ribonuclease
rRNA	ribosomal ribonucleicacid
rpm	revolutions per minute
S	Svedberg unit
^{35}S	ß emitting isotope of sulphur
SDS	sodium dodecyl sulphate

sec	second(s)
SSC	standard citrate saline
TEMED	NNN'N'-tetra-methyl-1,2-diamino-ethane
TCA	trichloroaceticacid
Tris	tris(hydroxymethy)-amino-methane
tRNA	transfer ribonucleicacid
U	unit
uv	ultraviolet
µCi	microcurie(s)
µg	microgram(s)
µl	microlitre(s)
µM	micromolar
µmol	micromole(s)
V	volt(s)
V_{max}	maximum velocity
v/v	volume per volume
w/v	weight per volume
N-t	amino-terminal
C-t	carboxy-terminal

STANDARD AMINO ACID ABBREVIATIONS

Ala	A	alanine
Asp	D	aspartic acid
Glu	E	glutamic acid
Phe	F	phenylalanine
Gly	G	glycine
His	H	histidine
Ile	I	isoleucine
Lys	K	lysine
Leu	L	leucine
Met	M	methionine
Asn	N	asparigine
Pro	P	proline
Gln	Q	glutamine
Arg	R	arginine
Ser	S	serine
Thr	T	threonine
Val	V	valine
Trp	W	tryptophan
Tyr	Y	tyrosine
Cys	C	cysteine

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CHAPTER 1

INTRODUCTION

1.1. NEMATODES

1.1.1. General Aspects

The Nematodes form a group of animals which, although known for more than 3000 years, have been greatly neglected until recently.

They are unquestionably the most abundant and successful group to have been produced by the evolutionary changes that have taken place among the pseudocoelomate animals.

Nematodes comprise the third largest phylum of invertebrates (15,000 species), being exceeded only by mollusca (100,000 species) and arthropods (about 850,000 species). In absolute numbers of individuals, they exceed all other metazoa. As parasites of animals, they exceed all other helminths combined. There are some 2000 known plant parasites, 4000 to 5000 animal parasites, and 8000 to 10000 marine, fresh-water, and terrestrial free-living species. Nematodes have been found from the deepest ocean floors to the highest mountains, from the Arctic to the Antarctic, and in soils as deep as roots can penetrate (Maggenti, 1982).

The phylum Nematoda comprises two classes: Secernentea and Adenophorea (Maggenti, 1982) (Table 1.1). Secernenteans are characteristically terrestrial (soil water); they are rarely encountered in fresh-water or marine habitats. It is within the Secernentea that most of the important parasites of humans, domestic animals,

plants and insects occur (Fig. 1.1). The aquatic, fresh-water and marine nematodes, are included in Adenophorea. A minority of parasites of plants and animals (e.g. *Trichinella, Trichurus*) are also included in this class (Fig. 1.1).

TABLE 1.1. A SCHEMATIC CLASSIFICATION OF NEMATODES.

Phylum: Nematoda (Nematoidea, Nema ta)

- Class: Adenophorea (Aphasmidia, Aphasmidea)
  - Subclass: Enoplia
    - Order: Enoplida
      - Isolaimida
      - Mononchida
      - Dorylaimida
      - ***Trichocephalida**
      - Mermithida
      - Muspiceida
  - Subclass: Chromadoria
    - Order: Araeolaimida
      - Chromadorida
      - Desmoscolecida
      - Desmodorida
      - Monhysterida
- Class: Secernentea (Phasmidia, Phasmidea)
  - Subclass: Rhabditia
    - Order: ***Rhabditida**
      - ***Strongylida**
      - ***Ascaridida**
  - Subclass: Spiruria
    - Order: ***Spirurida**
      - Camallanida
  - Subclass: Diplogasteria
    - Order: Diplogasterida
      - Aphelenchida
      - Tylenchida

*Bold type indicates orders containing human parasites.

Parasitic Nematodes are one of the major causes of infectious diseases which affect man and domestic animals. The cumulative effects of parasitism in terms of mortality, chronic disease and economic loss are

incalculably great. These effects undoubtedly play an important part in limiting the social and economic development of many countries in sub-tropical and tropical regions of the world (Stephenson, 1989; Latham, 1983).

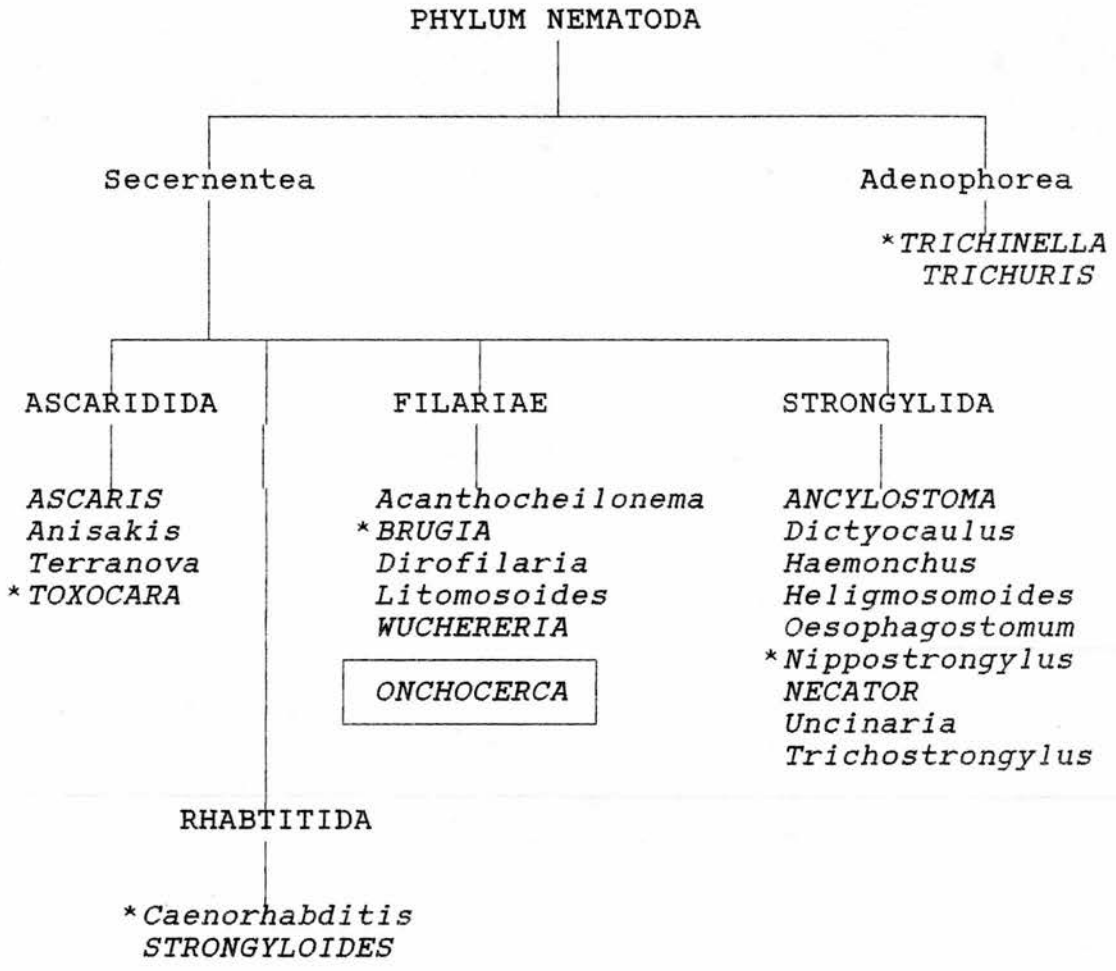


Fig. 1.1. Nematodes of major interest as medical or veterinary parasites, or as experimental models. Capital letters are used for genera containing parasites infective to humans, and the parasites most studies in each group are indicated by asterisks; Ascaridida, Rhabditida, and Strongylodida are orders of nematodes; Filariae are a suborder of Spirurida. Ancylostoma, Necator, and Uncinaria are collectively termed 'hookworms'.

Both the free-living and parasitic species have a highly conserved developmental cycle (Fig. 1.2), undergoing complete cuticle moults on four different occasions. Most parasitic worms are transmitted by the

mammalian hosts either as eggs from gastrointestinal worm forms or as emerged first stage larvae (L_1), as in the blood-borne microfilariae of filarial parasites. The form which is infective to mammalian hosts is generally moulted the third stage larva (L_3). Nevertheless, in some Ascarides, such as *Ascaris* and *Toxocara*, the infective stage is L_2. Between the L_1 and L_3 stages, most nematode organisms must undergo severe environmental shifts, moving from the homeostatic mammal to a free-living phase in the soil, or to an arthropod host, as in the case of vector-borne parasitisms.

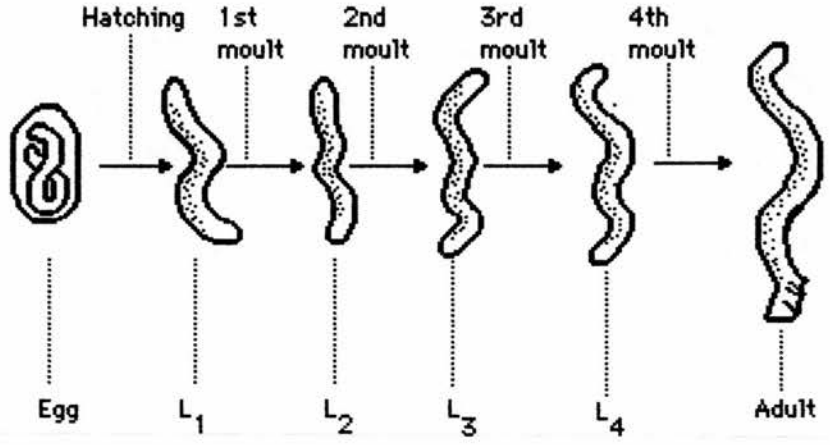


Fig. 1.2. A typical Nematode developmental sequence.

Exploitation of habitats other than those provided by the intestine of the host is widespread in the Nematode. Many species that live as adults in the intestine, such as *Ascaris*, hookworms, *Nippostrongylus* and *Trichinella*, undergo development in parenteral tissues. Other species are wholly confined to the tissues and have no contact with the intestine. The occupation of such niches within the host body requires particular adaptation

in reproductive biology, the parasite concerned no longer having direct access to the outside world. One way of solving this problem, as observed in *Capillaria* and *Trichinella*, is the production of eggs or cysts, which remain infective in the tissues even after the death of the host or when the host is eaten. Another solution, as observed in *Dracunculus*, is to break out of the surface of the host body in order to liberate larvae. In a major group of tissue-invading nematodes, the Filarioidea, the problem is solved by the involvement of a blood-feeding arthropod intermediate host in the life cycle (Fig. 1.3). The female worms liberate live embryos (microfilaria larvae) which circulate in the blood or accumulate in the skin. The arthropod takes up microfilariae when it feeds, provides an environment in which development into infectivity may occur, and then re-introduces the parasite into the vertebrate host during a subsequent blood meal. For the major filarial infections of man, the intermediate hosts are as follows:

Lymphatic filariases: *Wuchereria bancrofti, Brugia malayi* (transmitted by species of mosquito);

Onchocerciasis: *Onchocerca volvulus* (transmitted by species of the blackfly *Simulium*.

However, many other arthropods, including ticks and mites, can also transmit worms of this group.

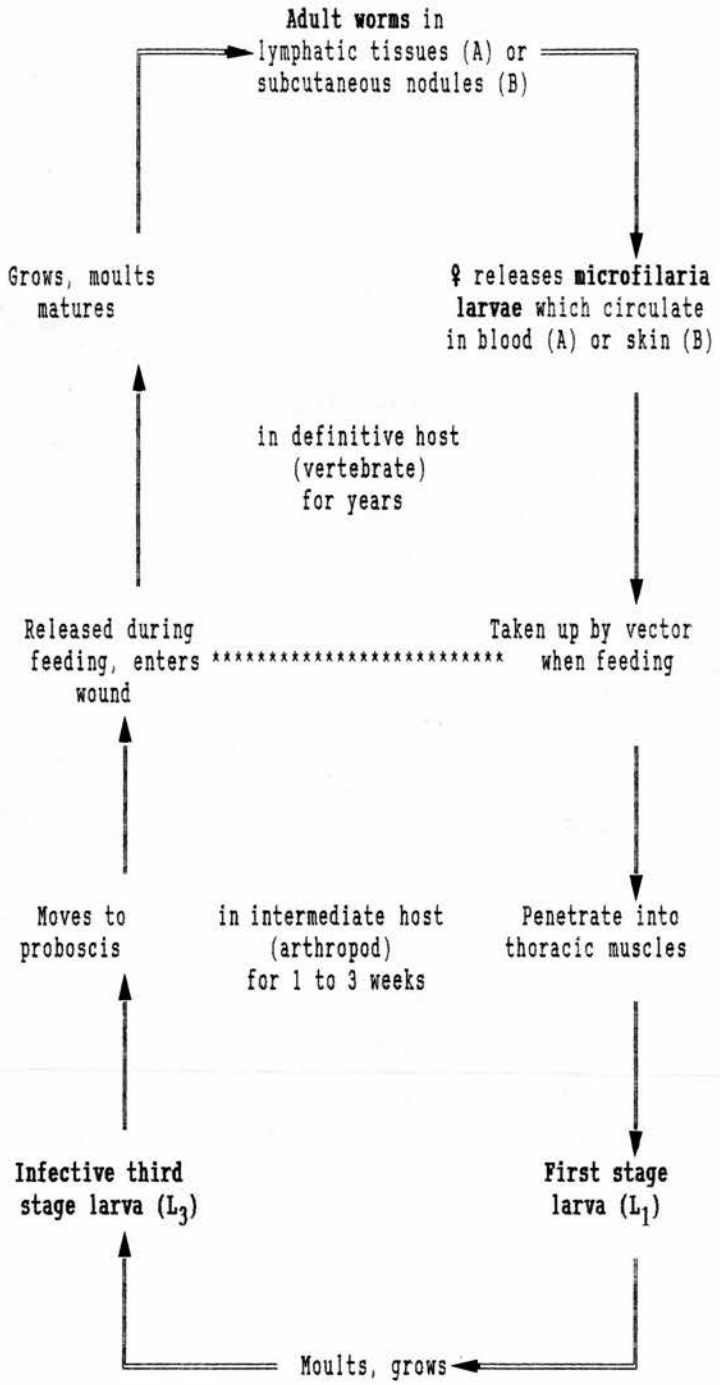


Fig. 1.3. Life cycles of human filarial nematodes. A = *Wuchereria* or *Brugia*, B = *Onchocerca*. The life cycles of the common laboratory models *Brugia pahangi* and *Dipetalonema vitae* are similar to A. *D. vitae* adults lie unencapsulated below the skin and their larvae are transmitted by ticks.

Nematode parasitisms therefore tend to be slowly developing diseases in which a cumulative body burden generates pathology, often exacerbated by inappropriate

immune responses from the human host. Rarely fatal, these parasites are maintained in the host population by the large numbers of infective stages transmitted by infected carriers over extended periods of time, and immunity to re-infection is generally weak if not altogether absent (Anderson & May, 1982).

1.1.2. Historical Background

Ancient people were probably familiar with larger nematodes. The earliest records mention the worms or contain recognizable allusions to them. For example, the Ebers Papyrus of 1550 B.C. described nematodes which we now know as *Ascaris lumbricoides* and *Dracunculus medinensis*. Some old Hebrew writings contained many references to what may be interpreted as parasitic diseases caused by nematodes, but specific mention of forms is lacking. Agatharchides (*circa* 180 B.C.) gave a description of *what was probably Dracunculus*. In addition, the Biblical Book of Numbers mentions the control and treatment of infections with this worm. It also seems that the "serpent of brass" placed upon a pole by Moses is connected with the traditional method of removing the worm from the subcutaneous tissues by winding it about a stick. Moses also wrote of a scourge that probably was caused by the Guinea worm. The Greeks made more definite references to threadworms and roundworms. For example, Hippocrates (*circa* 400 B.C.) and Aristotle (*circa* 350 B.C.) mention them in their writings. Roman physicians described and attempted to treat worm infections in various ways, and nearly all the references to nematodes till the end of the Middle Ages are concerned with disease and treatment. Herodotus (*circa* 130 A.C.) recommended the use of a seed (*Santonin*) for treatment. The Arabians Avicenna and Avenzoar, who kept parasitology alive during the Dark Ages in Europe, studied elephantiasis, differentiating it from leprosy (Foster 1965). The former recommended enemata for

the treatment of the pin worm *Enterobius vermicularis*, and, although occasionally, such a treatment is still used today. However, little progress in therapy was made for several hundreds of years, until the late sixteenth and the seventeenth centuries when new anthelminthics were introduced.

The free-living forms of Nematodes, only discovered in relatively recent times, were overshadowed by the economic and social importance of the parasitic ones. Even in the latter, there is a division into those infecting man and animals and those infecting plants. The first plant parasitic worms were not discovered until the eighteenth century, whereas the earliest record of forms found in man was more than 3000 years earlier than this. This difference in time meant that a considerable body of knowledge concerning nematode infections of man had been acquired before the full extent of the group was realised. The outstanding contributor was Leukart (circa 1870), who was one of those who did not restrict his attention to those forms of immediate medical interest. One important achievement of this era was the discovery by Manson (1878) of an insect intermediate host for *Wuchereria bancrofti* -- a finding important not only in itself, but also because it was the main stimulus for Ross' work on malaria (Manson-Bahr, 1963). Even now, our knowledge of the life histories of nematodes of medical importance is not complete and there is still active interest in this field.

1.2. FILARIAL WORMS

The family Onchocercidae, of the superfamily Filarioidea to which all the filarial parasites of man belong, comprises a well-defined group of tissue-dwelling nematodes all producing microfilariae which either circulate in the bloodstream or are present in the skin, and which develop into third-stage infective larvae in a

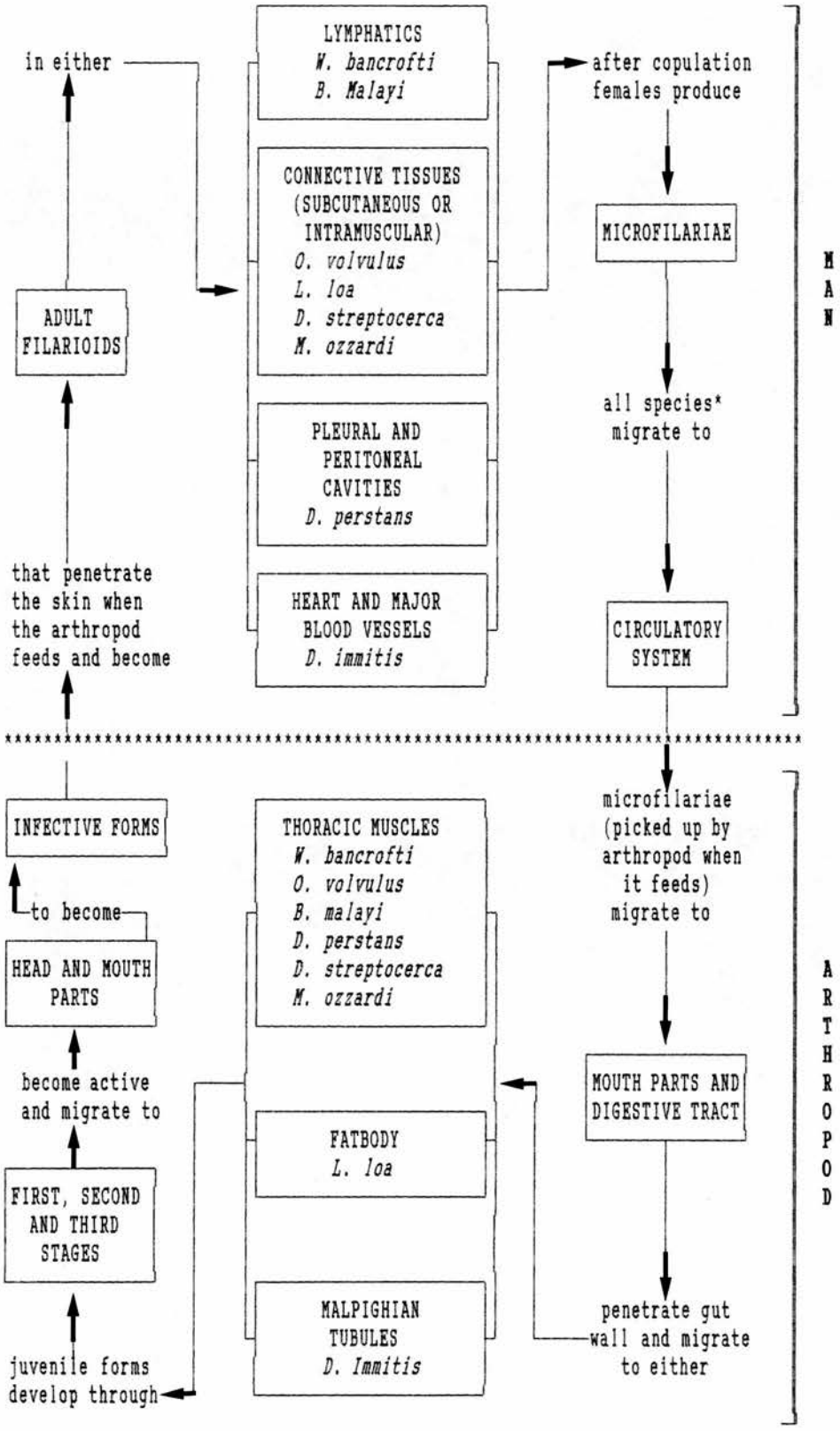
suitable arthropod vector (Fig. 1.4).

The generic taxonomy of the superfamily Filarioidea is not exact. There are about eleven species in six genera parasitic on man. The genus *Wuchereria*, found only in man, and the closely related *Brugia*, with species also parasitizing primates and other mammals, show a wide range of intraspecific variation. On the other hand, the genus *Loa* found in man and primates appears to consist of a single species as shown by experimental hybridization. The poorly described genus *Mansonella* occurs only in man but shows a wide range of intraspecific and probably specific variation. Two or perhaps three species of the recently re-erected genus *Tetrapetalonema* (Anderson and Bain, 1976) occur in man and higher primates. The genus *Onchocerca*, with one species in man and about 27 in ungulates, appears to consist of a rapidly speciating group of organisms with very variable morphological characteristics.

Wuchereria and perhaps *Mansonella* appear to be long-established human parasites, while *Brugia*, *Loa* and *Tetrapetalonema* might be primarily parasites of primates which became infective to man in geologically recent times. *Onchocerca* is apparently a capture from a herbivore although, unless an animal reservoir of *O. volvulus* remain to be found, this must have been long enough ago for subsequent speciation to occur.

The filarioids are an unusual group among parasitic nematodes in that their classification relies on factors additional to adult morphology: ecology, structure of the larval stages and life cycle can all be

FILARIOID DISEASES



* Except *O. volvulus* and *D. streptocerca* which migrate to tissue fluids.

Fig. 1.4. Relationships of life cycles of filarial parasites to mode of transmission.

important taxonomic criteria. Getting to know these parasites has been especially difficult because most (including *W. bancrofti* and *O. volvulus*, the two most important) cannot be maintained in laboratory animals. Thus, only limited amounts of parasite material have been available for study, and techniques for defining and differentiating substrains or even subspecies and species have been slow to develop.

1.3. ONCHOCERCIASIS

1.3.1. *O. volvulus*

O. volvulus belongs to a genus of filarial nematodes which remains poorly characterised, in spite of its importance to man, its sole host. It infects an estimated 18 million people and is associated with high rates of morbidity, including dermal atrophy and blindness (WHO, 1987).

The worm is prevalent in Africa, and in Central and South America, often affecting more than 50% of the inhabitants of towns and villages in endemic areas. The vector of *O. volvulus* is the blackfly *Simulium* spp. The breeding habitat of the blackfly is rivers and streams; hence the common term "river blindness" has been applied to onchocerciasis.

While members of the genus typically require 12-24 months to complete their life-cycle, three out of the four moults that occur during development take place within approximately two weeks. Two moults occur while microfilariae develop to infective, third-stage (L_3) larvae in the vector, while a third takes place in the definitive host 2-5 days after infection. Compressed into this early period of larval differentiation and growth are both crucial points of parasite transmission between the

invertebrate and vertebrate host (Duke, 1980; Bianco *et al.*, 1989).

1.3.2. Onchocerciasis in Man

The clinical pattern of onchocerciasis differs markedly in various parts of the world (see review by Braun-Munzinger & Southgate 1977). The most significant and obvious difference is in the prevalence of blindness due to onchocercal sclerosing keratitis in West Africa, where rates in the savannah regions may be up to three times as high as in the more southerly forest areas. Similarly, the clinical pictures of 'sowda' in Yemen and of Central American onchocerciasis in Guatemala differ markedly from anything seen in West or East Africa.

Irrespective of the strain of parasite the most severe clinical manifestations of onchocerciasis are due to reactions to the presence of the microfilariae. The adult worms are usually of secondary importance. This is in contrast to *Wuchereria* and *Brugia* infections where the main pathology is caused by adult worms.

In the African and Venezuelan forms of the disease the nodules are mostly distributed in the lower part of the body, whereas in Central America the nodules tend to be more in the upper part. In Guatemala, for example, the nodules are around the torso.

The anatomical distribution of adult worms is believed to determine the distribution of the microfilariae. This seems to be confirmed by the works of Kershaw *et al.* (1954), Nelson (1958), and Woodruff *et al.* (1966a) in Africa, Mazzotti (1951) in Mexico, Woodruff *et al.* (1966b), and De Leon & Duke (1966) in Guatemala.

It has been suggested that the distribution of

adult worms is related to the biting habits of the vectors. If this were true, the site of inoculation would also be of considerable importance in determining the pathogenicity in onchocerciasis. But this may not be the case. According to Duke (1968), the adult worms of *O. volvulus* always develop around the hip joint in chimpanzees irrespective of the site of inoculation. The parasite seems to have its own directional mechanism. This is also true for *O. gutturosa* in cattle, of which the microfilariae also have a well developed directional mechanism determining their final position in the skin. They migrate preferentially to the umbilicus where there are the best opportunities of being picked up by the vectors. This is a beautiful adaption to transmission, because more than 90% of the vectors bite around the umbilicus (Nelson, 1970).

1.3.3. Onchocerciasis in Domestic Animals

Although several species of *Onchocerca* are common in cattle and horses, they are seldom recognized as causes of ill-health in these animals. Bovine onchocerciasis is so unobtrusive that it goes unnoticed even in areas of high endemicity. A high rate of cryptic infections with *O. gutturosa* has been reported in cattle in Europe (Nelson *et al.*, 1966), and in many parts of the world (Gnedina, 1950; Supperer, 1952; Webber *et al.*, 1957); Clarkson, 1964). In Europe, both *O. cervicalis* and *O. reticulata* may infect horses without detection (Supperer, 1952; Nemeséri, 1956). But some species of *Onchocerca*, like *O. gibsoni* in cattle and *O. flexuosa* in deer are much more obtrusive. They produce nodular swellings under the skin which are easily seen when the carcasses are inspected. Perhaps the most obvious of all the species is *O. armillata* which occurs in the aorta of cattle (Abdel Malek, 1958; Patnaik, 1962).

On the whole, bovine and equine onchocerciasis have received very little attention. Even so, some important clinical observations have been made. For example, the adults of both *O. gutturosa* and *O. cervicalis* occasionally produce quite severe local reactions, yielding chronic inflammatory masses in the region of the cervical ligament (Nelson, 1970); the adults of *O. armillata* produce striking lesions in the intima and media of the thoracic aorta in cattle (Chodnik, 1958); *O. cervicalis* produces corneal, uveal and choroidal lesions similar to the lesions previously observed in human cases of ocular onchocerciasis (Lagraulet, 1962). Roberts (1963) has reviewed the problem of the etiological association of microfilariae in the eye with ocular lesions and he suggests that *O. cervicalis* can cause periodic ophthalmia in horses and that riboflavine deficiency may be a possible predisposing factor in much the same way as Vitamin A deficiency is thought by Rodger (1962) to accentuate ocular onchocerciasis in man.

Transmission has also received little attention. For example, the life cycle of *O. cervicalis* in *Culicoides nubeculosus* and *O. gutturosa* in *Simulium ornatum* has been described (Steward 1933, 1937; Supperer, 1952; Moignoux, 1952), but no detailed observations have been made on transmission; the development of *O. gibsoni* in *Culicoides pungens* has been described by Buckley (1938) in Malaya, but no detailed observations have been made on transmission either. Nevertheless, *O. gutturosa* can provide invaluable material for observations on the parasitology and transmission which are particularly relevant to onchocerciasis in man (Nelson *et al.*, 1968).

1.3.3.1. A Model System for *O. volvulus*

The above considerations suggest that research on species of *Onchocerca* which infect domestic animals can

contribute to the study of *O. volvulus*. A recent work suggests that *O. gibsoni* and *O. gutturosa* are closely related to *O. volvulus* (Lee Gill *et al.*, 1988). However, the karyotype of six species of *Onchocerca* indicates that *O. volvulus* is phylogenetically more closely related to *O. gibsoni* than to the other *Onchocerca* species examined including *O. gutturosa* (Post *et al.*, 1989). Thus, it seems that information learned about the biology of *O. gibsoni* may be applied to the human parasite, for which experimental material is not easily available. This would not only circumvent the problem of lack of availability of experimental material, *but would also provide an experimental system for biochemical analysis and drug screening.*

1.3.4. Geographical Distribution of *O. volvulus*

The filarial worm *Onchocerca volvulus* occurs throughout the greater part of tropical Africa, especially in the **rain forest regions** and the **savannah belt** that stretches for more than 4000 miles from the Atlantic coast of Senegal to the Indian Ocean in Tanzania. This is believed to be the original home of the disease, the great endemic area where more than 18 million people are infected (Fig. 1.5). There are many areas in the savannah regions of Africa where 30% of the population may have impaired vision as a result of onchocerciasis and the blindness rates in the adult population may be over 10% (Nelson, 1970).

The discovery by Morgan (1958) of onchocerciasis in the villages along the Nile near the Egypt-Sudan border was quite unexpected; the landscape epidemiologists would hardly have suspected that transmission could have occurred in this dry, arid desert region of the Sahara.

Another unusual and little studied focus of

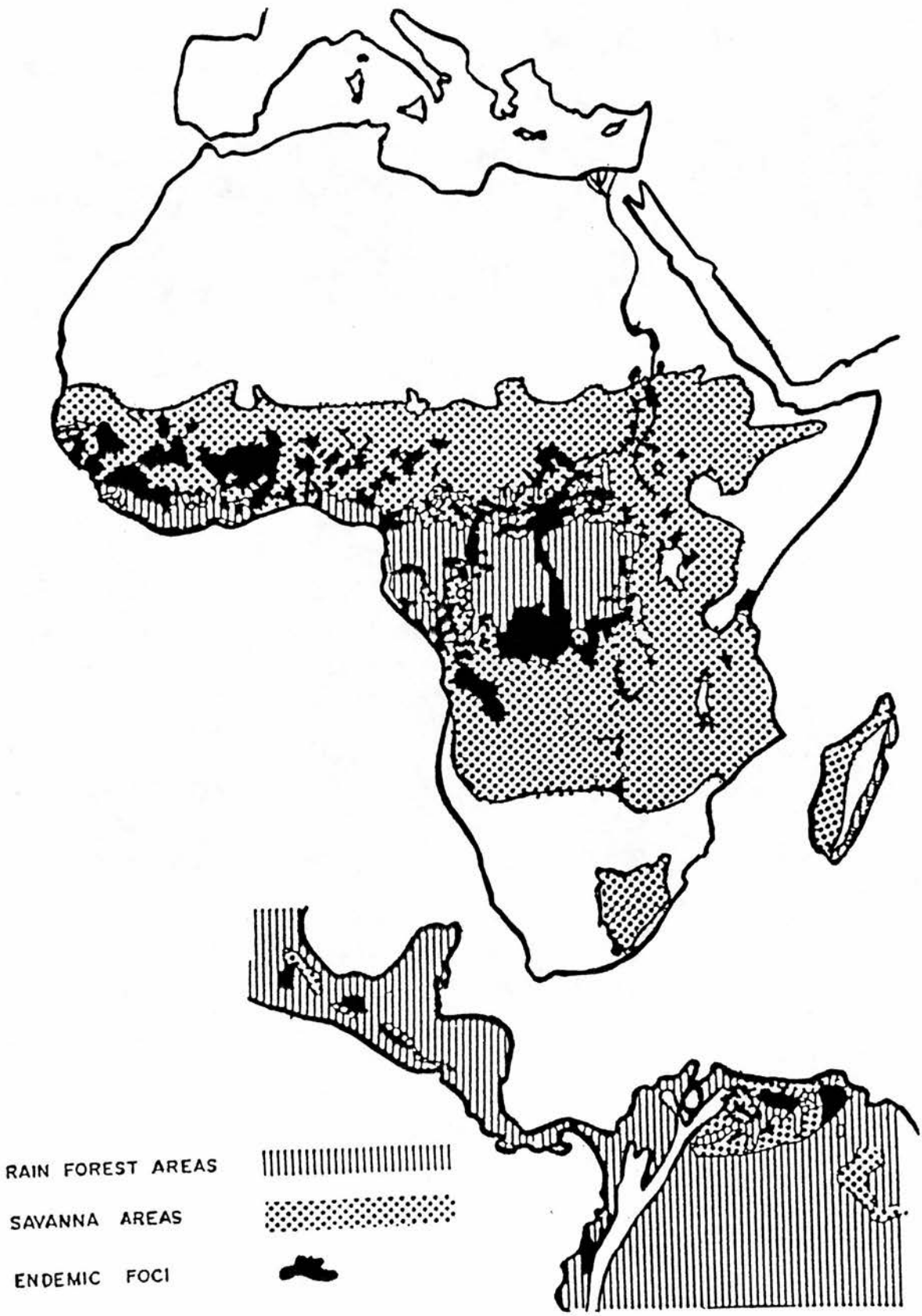


FIG. 1.5. The geographical distribution of onchocerciasis in man.

onchocerciasis occurs in the Yemen Arab Republic (North Yemen). For many years the infection was believed to differ both epidemiologically and clinically from the forms seen in West and East Africa and the Americas. However, Buettner *et al.* (1982) have redefined the clinical syndrome of "sowda", and related it to parasitological and immunological parameters in the affected population. They have also clearly demonstrated the gaps which remain to be filled in our knowledge of transmission aspects of epidemiology in this country, although Garms & Kerner (1982) have incriminated *S. damnosum* sensu latu as one vector in this country.

Although the broad outlines of the geographical distribution of *Onchocerca volvulus* have been generally well-known for a number of years, several new foci have been described recently. For instance, onchocerciasis has been described in Ecuador (Arzube *et al.*, 1981; Guderian *et al.*, 1982; Arzube, 1982). Other foci were found in Central and South America: Guatemala, Mexico, Venezuela, Colombia, Brazil (Table 1.2).

Further studies will no doubt show that onchocerciasis is far more widespread than is indicated in Fig. 1.5. The disease may not occur in Arctic regions but there is a possibility that it will be found in other areas, especially in Asia and South America where the rivers flowing from the great mountain chains are often ideal breeding places for its vectors.

TABLE 1.2. GEOGRAPHICAL DISTRIBUTION OF *O. volvulus*

Region	Country	Reference
Africa	Kenya	Preston (1935) Buckley (1949) McMahon et al. (1958
	Uganda	Nelson (1958) Gibbins (1939) Barnley (1949) Barnley & Prentice (1958) McCrae & Prentice (1963) McCrae (1965)
	Congo	Hisette (1932) Lebied (1950
	Sudan	Bryant (1935) Lewis (1948) Lewis (1953) Lewis (1960)
	Tanzania	Raybould (1957) Wegesa (1968) Hausermann (1966)
	Nigeria	Crosskey (1957) Crosskey & Crosskey (1959) Davies (1963) Davies (1965)
	Ghana	Crisp (1956) Marr & Lewis (1964) Noamesi (1964)
	Guinea	Garms & Post (1966)
	Egypt	Morgan (1958)
	Former British West African Colonies	Ridley (1945) Budden (1957) Rodger (1959)

Continued

TABLE 1.2. CONTINUATION

Region	Country	Reference
Africa	Former French Colonies	Puyuelo & Holstein (1950) D'Aussy et al. (1958) Quéré et al. (1963) Toussaint & Danis (1965) Lagraulet et al. (1967) Ovazza (1953) Grenier et al. (1955) Le Berre (1966)
Near East	Yemen	Fawdry (1957)
Central and South America	Colombia	Assis-Masri & Little (1965)
	Guatemala	Pacheco-Luna (1918) Robles (1919) Gibson & Dalmat (1952) Dalmat (1955) De Leon (1957) De Leon (1963) Gonzalez-Peralt (1983) Brandling-Bennett et al. (1981)
	Mexico	Hoffman (1930)
	Venezuela	Lewis & de Aldecoa (1962)
	Ecuador	Arzube et al. (1981) Guderian et al. (1982) Arzube (1982)
	Brazil	Shelley & Luna-Dias (1980) Cerqueira (1959)

1.3.4.1. *O. volvulus*: Geographical Variants

Onchocerca volvulus is not a uniform species; there are distinct strains or biological variants of the parasite each with its own inherent properties which determine the pattern of transmission and the subsequent clinical picture in man (Duke, 1974). As long ago as 1919 Brumpt noted striking differences in the epidemiology of onchocerciasis in Central America and Africa and De Leon and Duke (1966) suggest that the Central American and

African parasites are at least distinct strains or races of *O. volvulus*. Apart from the differences between the African and American forms of *O. volvulus* there is also a good deal of clinical and pathological evidence suggesting that there are several variants of *O. volvulus* itself (Woodruff *et al.*, 1966a, b): the savannah and forest forms in West Africa differ quite markedly in their vector infectivity, in their infectivity to chimpanzees and in their general epidemiology and clinical manifestations (Duke *et al.*, 1966; 1967; Lewis and Duke, 1966; Duke, 1966, 1967); there are also marked differences in the clinical manifestations of onchocerciasis in West and Eastern Africa.

New molecular-genetic data on the river-blindness parasite, reported by Erttmann *et al.* (1987), Harnett *et al.* (1987) and Meredith *et al.* (1989), distinguish between two strains of the parasite and show a clearly marked difference in the DNA of the forest and savannah strains. These workers isolated a DNA probe, pFS-1, comprising 153 nucleotides from a forest strain which specifically hybridizes with the forest strain DNA but not with the savannah one, and most importantly, not with DNA from man or the *Simulium* vector.

Several other groups have reported the cloning and characterisation of repeated DNA sequences from *Onchocerca* species with potential value for species differentiation. Thus, Perler & Karam (1986) have isolated a sequence (designated pOv26) which specifically hybridises with *O. volvulus* and *O. gutturosa* DNA; Post & Crampton (1987), have identified a sequence which hybridises with *O. gutturosa, O. gibsoni* and *O. ochengi* DNAs; and Murray *et al.* (1988) have found another specific probe for *O. armillata*.

Biochemical evidence for the existence of forest

and savannah strains was first provided by Omar *et al.* (1982) and Bryceson (1976) and more recently supported by a comparison of isoenzyme patterns (Flockhart *et al.*, 1986) and antigenic profiles (Lucius *et al.*, 1987).

1.3.5. The Vectors of *O. volvulus*

It has been found that *O. volvulus* is transmitted by *Simulium* flies (Blacklock, 1926). Long before Europeans arrived in Africa, the local people suspected that insects were concerned with transmission of the disease. For example, the people inhabiting the "valley of the blind" in Kenya recognized the skin disease and the eye lesions and when asked by an agriculturist how they contracted the disease they took him to a river and showed him a biting fly which was subsequently identified as *S. neavei* (Dry, 1921).

The species of simuliids found naturally infected in Africa are *S. damnosum*, *S. neavei*, and *S. woodi*. But *S. damnosum* is the main vector of *O. volvulus* in this continent. *S. damnosum* breeds on vegetation and on rocks in a great variety of river systems, ranging from great rivers such as the Nile and the Congo to the seasonal streams on the edge of the desert in the northern savannah regions. This is by far the most important vector.

Members of the *S. neavei* complex are vectors of onchocerciasis in limited areas in the eastern Congo, in Uganda, Kenya, Tanzania and Ethiopia. The *S. neavei* complex is a distinct group which breeds mainly in small streams in highland areas and nearly all species have an obligatory association with fresh water crabs of the genus *Potamonautes*. It is not known where the females of *S. neavei* and other members of this complex deposit their

eggs, but the larvae and pupae are found only on crabs. This phoretic association was first demonstrated with *S. neavei* by Van Someren and McMahon (1950) in Kenya, and subsequently in *S. nyasalandicum, S. woodi, S. goinyi, S. hightoni* and *S. ovazza* (Lewis and Hanney, 1965); *S. neavei* is the main vector throughout East Africa, but in Tanzania it is replaced by the closely related *S. woodi* (Raybould, 1967).

The vector in the Yemen is unknown. The work of Buettner *et al.* (1982) has demonstrated the gaps which remain to be filled in the knowledge of transmission aspects of epidemiology in this country, although Garms & Kerner (1982) have described *S. damnosum* as one vector in Yemen.

In Guatemala and Mexico, three species of *Simulium* have been described as vectors, namely *S. callidum, S. metallicum* and *S. ochraceum* (Dalmat, 1955). The studies by De Leon and Duke (1966) suggest that in Guatemala *S.ochraceum* is by far the most important vector. It is markedly anthropophilic and breeds in the numerous small streams and rivers flowing through the thickly wooded volcanic slopes of this country. Even the most minute trickles may form breeding places, thus making control extremely difficult (Crosskey, 1968). Both *S. metallicum* and *S. callidum* are strongly zoophilic and it is doubtful whether they are of much importance as vectors in this area. However, in Venezuela onchocerciasis has now been recorded in nine states and here *S. metallicum* is thought to be the main vector (Lewis and de Aldecoa, 1962; Rivas *et al.*, 1965; Arends, 1966). *S. metallicum* is an infrequent biter producing a situation similar to that seen in areas of relatively low endemicity in Africa. The focus discovered in Colombia is not thought to be continuous with that in Venezuela, and the vector is unknown, although Lewis and Lee-Porter (1964) have

recorded several man-biting species in this country.

1.3.6. *Simulium* Control

The vectors of onchocerciasis can be controlled either by modifying the habitat or by destroying some stage in the life cycle of the fly. One of the earliest successful control projects affecting the habitat was carried out in Kenya (Buckley, 1951). This project demonstrated that the method of selective bush clearing was effective against *S. neavei*. But the method was expensive and never adopted on a wide scale. In fact, it was soon superseded by DDT, which was shown to be effective against African simuliids by Garnham & McMahon (1947).

But modification of the habitat can still be an effective control measure in some situations. For example, the construction of dams usually creates lakes where there were formerly cascades and waterfalls: this can eliminate breeding places for many miles upstream. Downstream, the engineering work can produce the opposite effect, for obstruction of river flow can provide extra breeding places for simuliids (Burton & McRae, 1965; Garms & Post, 1966). But this is usually of concern only in minor projects, because major engineering works always provide strict control of the water flow at the outlets of the dams, and this in turn provides an easy method of applying larvicides downstream. In Uganda, the incorporation of a DDT dosing device into Jinja dam was able to eliminate *S. damnosum* from an area of at least 1600 square miles (Barnley, 1953; 1958).

In the Jinja focus, however, *S. damnosum* is confined to the Nile and there is little danger of reinfestation from tributaries. Elsewhere in Uganda and in other parts of Africa, control has been less effective

because breeding also occurs in several small streams. Control can only be effective by the application of larvicides at many dosing points and at much more frequent intervals. Even so, there has been some success in controlling *S. damnosum* by dosing the rivers with DDT as a larvicide, mainly at Mayo Kebbi in Chad (Taufflieb, 1956), at Abuya and Kainji in Nigeria (Davies *et al.*, 1962; Davies 1963; 1965; McMahon, 1967), and also in Northern Ghana and Upper Volta (Brown, 1962; McMahon, 1967).

In an earlier scheme, *S. damnosum* was controlled on the Congo river around Kinshasha by spraying with DDT from the air (Wanson *et al.*, 1949; Lebrun, 1954). But this method has so far proved either too expensive or impracticable in the main onchocerciasis areas of Africa and South America.

Total eradication of a vector simuliid has only been achieved in Kenya, where McMahon *et al.* (1958) eliminated *S. neavei* from an area of 6000 square miles. The success of the scheme has been confirmed by Nelson & Grounds (1958), and Roberts *et al.* (1967). The scheme was facilitated by the following features: i) *S. neavei* has a very limited flight range; ii) the affected area was geographically isolated; iii) it was often possible to transport the insecticide to dosing points by road; iv) the streams were all perennial so that the application of insecticide could be made throughout the year; v) the obligatory association of *S. neavei* with crabs made it possible to assess the effect of the larvicides by the sensitive method of examining crabs for larvae and pupae (Nelson, 1970).

The problems of eradication are much more formidable in areas where the vector is *S. damnosum*. This species is found throughout an area of several million

square miles, it has a flight range of at least 100 km, and in the savannah region a proportion of the flies may survive during the dry season (Ovazza *et al.*, 1965; Noamesi, 1966). In Central and South America there are equally formidable problems.

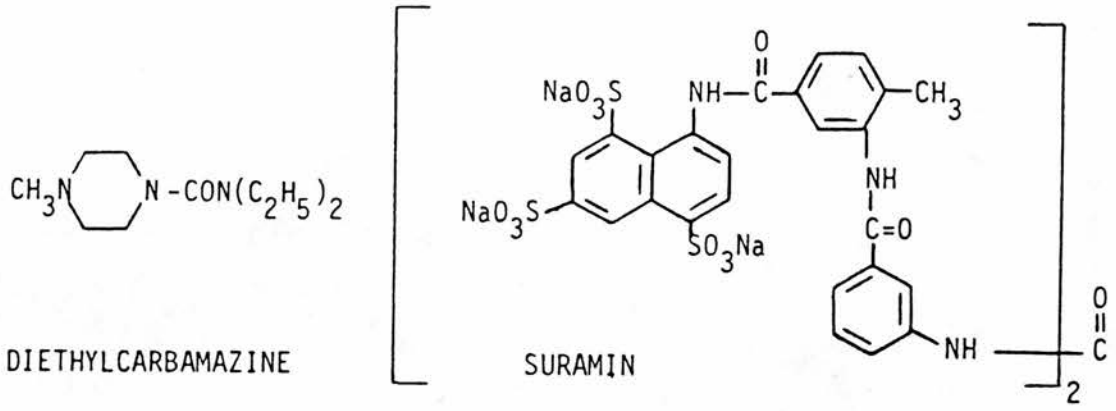
In all the endemic areas, total eradication of the vector may be impossible, but much can be done to limit the intensity of transmission. This would bring immediate benefit to the younger generations in these areas.

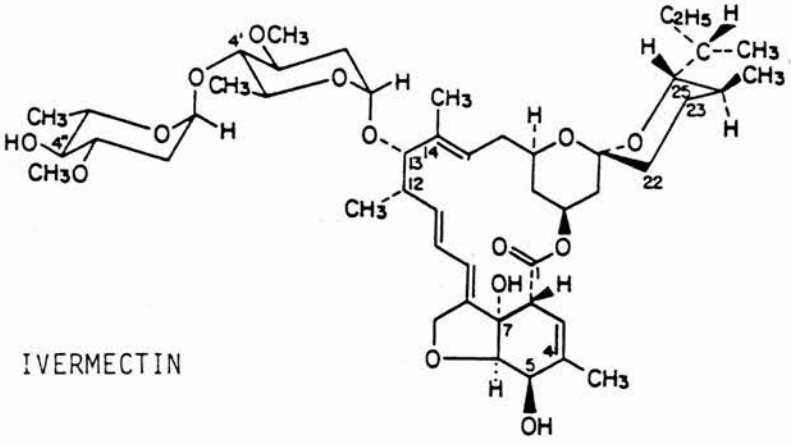
None of the vectors of onchocerciasis has so far developed resistance to DDT. But there is some evidence that some other species of simuliids can develop some degree of resistance (Suzuki *et al.*, 1963). The concentration of DDT to eliminate *Simulium* larvae has a negligible effect on man and the environment. However, the use of residual insecticides in streams and rivers is dangerous, and many of the unforeseen hazards have been denounced by Carson (1962).

1.3.7. Chemotherapy of Onchocerciasis

The chemotherapeutic treatment of filarial infections has never been satisfactory. Forty years ago Findlay (1950) recorded that in efforts to find an effective drug an enormous number of compounds had been tried, including such diverse agents as iodine, salts of gold, copper, tin, zinc, lead, mercury, arsenic and antimony, acridine derivatives, emetine, cobra venom and picric acid.

Only a few drugs have been used without excessive direct toxicity in human onchocerciasis (Goodwin, 1984) (Fig. 1.6). These include, for example, **diethylcarbamazine (DEC)**, **suramin**, **benzimidazoles**,





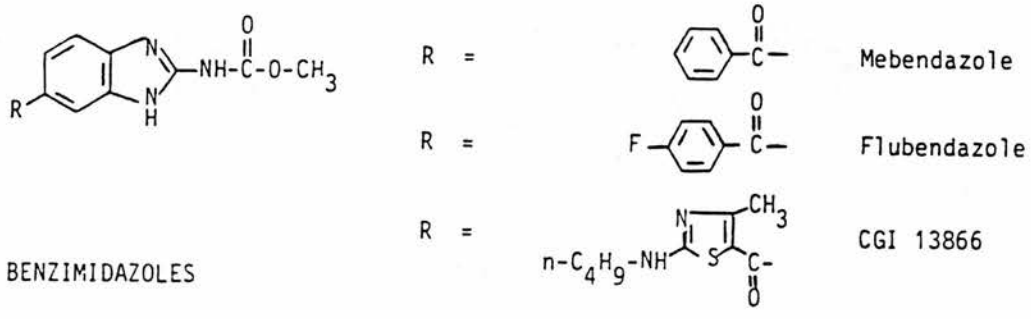


FIG. 1.6 Antifilarials.

ivermectin, and **isothiocyanates** and their derivatives. But all of them except ivermectin are followed by a post-treatment reaction (termed Mazzotti reaction in onchocerciasis) which results from the immunological inflammatory mechanisms activated in the process of clearing and killing the skin-swelling or blood-borne microfilariae.

Diethylcarbamazine is predominantly a microfilaricide affecting the neuromuscular system of the parasites and promoting cellular cytotoxicity mediated by immune factors. **Suramin** is macrofilaricidal, has a narrow therapeutic index and damages the intestinal epithelium of the worms. **Benzimidazoles** bind to tubulins and inhibit their assembly into microtubules. **Ivermectin** is an extremely potent microfilaricide in onchocerciasis; it augments immune responses and impairs the neuromuscular function of the parasites, leading to paralysis. **Isothiocyanates** and their derivatives are both microfilaricidal and macrofilaricidal and affect the energy metabolism of the parasites.

The precise primary effects of the old and new antifilarials are far from being understood. The possible sites of action are summarized in Table 1.3. Some information is available on the effects of the drugs on the carbohydrate and folate metabolism of the parasites (Hawking, 1978a; b; Van den Bossche *et al.*, 1982; Subrahmanyam, 1983, 1987; Goodwin, 1984; Mackenzie, 1985; Campbell, 1985). It is therefore pertinent to review briefly the carbohydrate and folate metabolism of the parasites and the mode of action of some drugs.

TABLE 1.3. Possible Sites of Action of Antifilarial Compounds

Antifilarial agent	Sites of action
Diethylcarbamazine	Neuromuscular system, cuticular surface, carbohydrate and folate metabolism, host immune factors
Suramin	Carbohydrate and folate metabolism, protein kinases, intestinal epithelium
Ivermectin	Neuromuscular system, host immune factors
Benzimidazoles	Assembly of microtubules
Isothiocyanates and derivatives	Cuticular surface, carbohydrate metabolism, cyclic AMP phosphodiesterase, 5'-nucleotidase, aminoacyl-tRNA ligases
Levamisole	Neuromuscular system, carbohydrate metabolism
Arsenicals	Carbohydrate metabolism, intestinal epithelium, glutathione metabolism
Antimonials	Carbohydrate metabolism

1.3.7.1. Carbohydrate metabolism

Filarial parasites have active glycogenic and glycolytic pathways and a somewhat subdued citric acid cycle. Most of the enzymes of these pathways have been identified (Ramp & Kohler, 1984; Barret *et al.*, 1986). The parasites metabolize glucose to lactate, acetate and carbon dioxide. The pathways of carbohydrate metabolism in filarial parasites are summarized in Fig. 1.7.

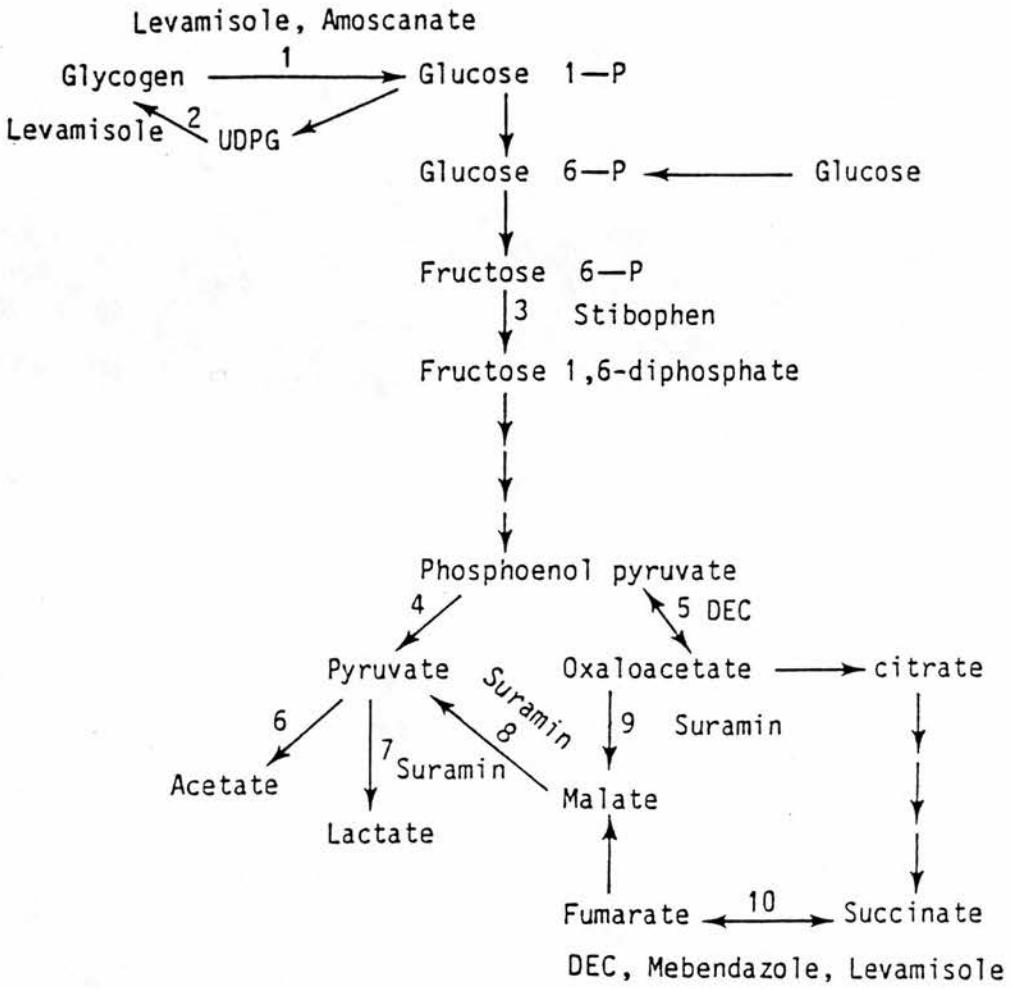


FIG. 1.7 Enzymes of carbohydrate metabolism in filariids, with sites of action of antifilarials indicated. UDPG, UDPglucose; DEC, diethylcarbamazine. 1, glycogen phosphorylase (EC. 2.4.1.1); 2, glycogen synthase (EC 2.4.1.11); 3, phosphofructokinase (EC 2.7.1.11); 4, pyruvate kinase (EC 2.7.1.40); 5, phospho*enol*pyruvate (PEP) carboxykinase/PEP carboxylase (EC 4.1.1.32); 6, pyruvate dehydrogenase complex; 7, lactate dehydrogenase (EC 1.1.1.27); 8, 'malic' enzyme (EC 1.1.1.40); 9, malate dehydrogenase (EC 1.1.1.37); 10, succinate dehydrogenase/fumarate reductase (EC 1.3.99.1).

1.3.7.2. Folate Metabolism

Several enzymes of folate pathways have been identified in filarial parasites (Jaffe, 1980) (Fig. 1.8). Analysis of the different steps of folate metabolism in the parasites reveals certain basic differences from those operating in vertebrates. The enzyme 5,10-methylene FH_4 reductase catalyses the irreversible formation of 5-methyl FH_4 from 5,10-methylene FH_4 in almost all vertebrate tissues. However, in filarial parasites this enzyme operates preferentially in the reverse direction, favouring the formation of 5,10-methylene FH_4. The parasites possess 10-formyl FH_4 dehydrogenase, which catalyses the deformylation of 10-formyl FH_4, thus regulating the endogenous concentration of FH_4 cofactors. This enzyme is more active in *B. pahangi* and *D. immitis* than in mammalian liver (Jaffe, 1980).

Another enzyme of interest is serine hydroxymethyl-transferase [glycine hydroxymethyl-transferase], which catalyses the synthesis of 5,10-methylene FH_4 from tetrahydrofolate (FH_4) and requires pyridoxal phosphate. There is a virtual block in the development of infective larvae of *L. carinii* to the adult stage in pyridoxine-deficient rodents, possibly because of interference with folate metabolism (Subrahmanyam 1983).

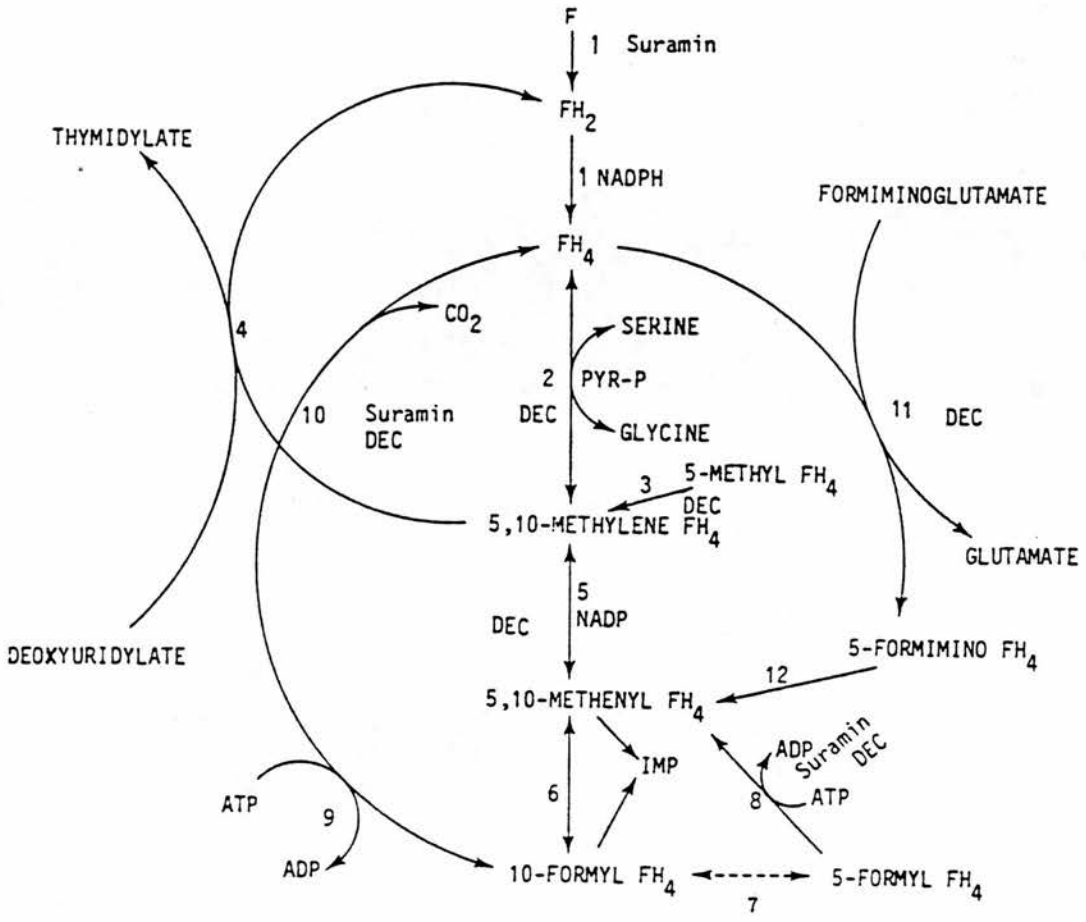


FIG. 1·8 Folate metabolism in filariids, with sites of action of antifilarials indicated. F, folate; FH_2, dihydrofolate; FH_4, tetrahydrofolate; PYR-P; pyridoxal phosphate; IMP, inosinate; DEC, diethylcarbamazine. 1: FH_2 reductase (EC 1.5.1.3); 2: serine-hydroxymethyltransferase [glycine hydroxymethyltransferase] (EC 2.1.2.1); 3: 5,10-methylene FH_4 reductase ($FADH_2$) (EC 1.7.99.5); 4: thymidylate synthase (EC 2.1.1.45); 5: 5,10-methylene FH_4 dehydrogenase ($NADP^+$) (EC 1.5.1.5); 6: 5,10-methenyl FH_4 cyclohydrolase (EC 3.5.4.9); 7: putative 5-formyl-, 10-formyl FH_4 mutase; 8: 5-formyl FH_4 cyclo-ligase (EC 6.3.3.2); 9: formate–FH_4 ligase (EC 6.3.4.3); 10: 10-formyl FH_4 dehydrogenase (EC 1.5.1.6); 11: glutamate formiminotransferase (EC 2.1.2.5); 12: 5-formimino FH_4 cyclodeaminase (EC 4.3.1.4).

1.3.7.3. Diethylcarbamazine (DEC)

The action of piperazine derivatives was first studied by Hewitt *et al.* (1947), who showed that the addition of a carbethoxy or carbonyl radical in position 1, with various substituents in position 4 of the piperazine nucleus gave rise to a series of active compounds. 1-Diethylcarbamyl-4-methylpiperazine (diethylcarbamazine; DEC) was selected as the most promising derivative and has since been very widely used in the treatment of human filariasis. Like suramin, it frequently causes unwanted side effects, the most serious being the Mazzotti reaction. Neither DEC nor suramin is suitable for the mass treatment of onchocerciasis, but DEC has been used in the mass treatment of lymphatic filariasis because the Mazzotti-type reactions are generally less severe.

DEC is effective as a microfilaricidal and probably macrofilaricidal agent when administered clinically in low doses. DEC is capable of killing the adults of *W. bancrofti, B. malayi* and *B. timori* in many patients treated with conventional drug regimens, but not adults of *O. volvulus* (Mackenzie, 1985).

Although DEC is one of the oldest drugs in the treatment of filariasis, its precise mode of action is still not clear. Piperazines, to which class DEC belongs, cause hyperpolarization of the muscle membranes of nematodes with concomitant flaccid paralysis. DEC inhibits acetylcholinesterase in *O. volvulus* (Walter, 1981), but it has virtually no effect on adult *Onchocerca* (Goodwin, 1984).

DEC alters glucose uptake in *L. carinii* and inhibits phosphoenolpyruvate carboxykinase, fumarate reductase and succinate dehydrogenase, which suggests that

the phosphoenolpyruvate-succinate pathway is one of the targets (Fig. 1.7). DEC also inhibits filarial 5,10-methylene FH_4 reductase, serine hydroxymethyltransferase and glutamate formiminotransferase (Fig. 1.8).

The extensive literature on the use of DEC and suramin has been surveyed by Hawking (1978a, b) and Mackenzie (1985), and current views on the difficult problem of using these drugs in the treatment of onchocerciasis have been summarized by Duke *et al.* (1981).

1.3.7.4. Suramin

The first major advance in the chemotherapeutic treatment of onchocerciasis was given by Van Hoof *et al.* (1947), who noticed that in patients with sleeping sickness treated with suramin, *O. volvulus* often disappeared. Suramin proved to be an effective macrofilaricide. It affects the epithelium of the intestinal lumen of the worms and alters the absorptive and permeability characteristics of the gut (Howells *et al.*, 1983).

Suramin has a strong interaction with glycolytic pathway enzymes (Fig. 1.7), and specific inhibition of the parasite's NADP-linked malic enzyme has been demonstrated in both *O. volvulus* and *D. immitis* (Walter & Albiez, 1981). A suramin-sensitive protein kinase has been demonstrated in *O. volvulus*, suggesting that this drug may interfere with the phosphorylation of regulatory proteins (Walter & Schulz-Key, 1980). Suramin also inhibits the dihydrofolate reductase of *O. volvulus* and the NAD-dependent 10-formyl FH_4 dehydrogenase of *B. pahangi*. Jaffe (1980) considers that the clinical efficacy of suramin against *Onchocerca* is due to inhibition of the dihydrofolate reductase of this parasite. However, much more should be known about the nucleotide synthesis by

this organism before the dihydrofolate reductase can be considered a suitable target enzyme (Fig. 1.8).

1.3.7.5. Ivermectin

Avermectins are macrocyclic lactones which belong to a family of natural products from the mycelia of *Streptomyces avermitilis*. Ivermectin is a semi-synthetic analogue and is a mixture of the B_1a (R = C_2H_5) and B_1b (R = CH_3) derivatives (Fig. 1.6). Ivermectin has a broad spectrum of antiparasitic activity and has, for several years, been used in veterinary medicine as an intestinal anthelmintic and for the control of arthropod ectoparasites.

Ivermectin is as effective as DEC in killing microfilariae, but induces much less of a Mazzotti reaction, with little or no inflammatory side-effects in the eyes of infected patients (Greene *et al.*, 1985). It was tested on patients with *O. volvulus* infections (Aziz *et al.*, 1982) and a single dose of 50 to 200 μg reduced the skin microfilariae by 90% in the course of a week and there was no Mazzotti reaction, except in a few cases in which the reaction was very mild. The effect on concentrations of skin microfilariae appeared to last for up to 3 months; in one case, although the drug had no direct action on microfilariae in the cornea or anterior chamber of the eye, the organisms gradually disappeared from them during the month following treatment. It is not yet known whether the drug has any effect on the adult worms and since the mechanisms underlying the triggering of the Mazzotti reaction by DEC have not yet been clarified, it is unclear how ivermectin avoids triggering this reaction while still effectively killing microfilariae.

All the other drugs -- DEC, suramin, and even mebendazole - that bring about the death of microfilariae give rise to a more or less severe Mazzotti reaction; the reason why ivermectin does not is an intriguing problem for future research. Unless difficulties arise with its more widespread use, this drug could have a place both for individual and for large-scale treatment of onchocerciasis (Goodwin, 1984).

The main action of ivermectin on nematodes seems to be on their nervous system. It promotes γ-aminobutyric acid (GABA) release and its binding to postsynaptic receptors, thereby affecting GABA-mediated neurotransmission. The GABA-mediated effects can be reversed by picrotoxin. Campbell (1985) reveals that the blockage of inhibitory neuromuscular transmission in *Ascaris suum* by ivermectin is not reversible by picrotoxin, which suggests other mechanisms of action.

Ivermectin inhibits the activation of protein kinase C from rat brain, which regulates the enzymes of glycolysis and related pathways. It is relatively safe to higher animals including humans because it does not pass readily through the blood-brain-barrier (Subrahmanyam, 1987).

1.3.7.6. Benzimidazoles

Benzimidazoles (BZs) represent a class of low-dose broad-spectrum anthelmintics with a high therapeutic index, widely used for the control of nematode infections in animals (Prichard, 1978; Zahner *et al.*, 1988; Gallay and Schweizer, 1981). It is an antimitotic drug, known to bind to tubulin and inhibit the growth of many lower eukaryotes, such as fungi (Davidse & Flach, 1977).

Mebendazole (MB), a representative filaricidal

benzimidazole, interferes with embryogenesis and is microfilaricidal in human onchocerciasis (Awadzi *et al.*, 1982; Kale, 1982; Rivas-Alcala *et al.* 1981a, b). The embryostatic effect of mebendazole was confirmed in human onchocerciasis at Tamale (Awadzi *et al.*, 1982), in Ibadan (Kale, 1982), and in Mexico (Rivas-Alcala *et al.*, 1981a, b).

Mebendazole also inhibits *in vitro* and *in vivo* glucose uptake by helminths which is followed by an enhanced utilization of endogenous glycogen and reduced glycogenesis. It also diminishes ATP synthesis and/or the turnover of adenine nucleotides. Mitochondrial electron transport, especially by the fumarate reductase system, is inhibited by several benzimidazoles in nematodes (Subrahmanyam, 1987, Van den Bossche *et al.*, 1982).

However, the primary targets of benzimidazoles are tubulins; by binding to these proteins the drugs interfere with the assembly of microtubules (Van den Bossche *et al.*, 1982). Lacey (1988) suggests that the effects of BZs on other processes in the cell, such as transport and anaerobic metabolism, probably result from the inhibition of one or more of the functions of tubulin. Thus, the disappearance of tegumental or intestinal cells consequent to inhibition of tubulin assembly in parasitic worms might result in a block of transport of secretory vesicles, decreased digestion and reduced absorption of nutrients. The specific interaction of benzimidazole with tubulin will be discussed in section 1.4.1. and in more detail in chapter 3.

The above situation led the Special Programme Scientific Working Group on Filariasis to set forward its basic chemotherapeutic aims: to improve the use of existing filaricides and to find new ones (WHO, 1979). As a result, the main needs are: a) to find means of reducing

the inflammatory reactions that occur in response to the death of filarial worms, particularly tissue-dwelling microfilariae; b) to determine the safest and most practical schedules for the immediate treatment of patients with ocular onchocerciasis who are at high risk of blindness; c) to find and develop new macrofilaricidal compounds, the most pressing need being for a safe drug which will kill or permanently sterilize the adult worms of *O. volvulus*, but which does not at the same time cause tissue damage as a result of allergic responses to the rapid destruction of microfilariae; and d) to determine the most practical dosage schedules for large scale treatment to control lymphatic filariases.

More than 10,000 compounds have now passed through the screens set up by the Special Programme, and several promising groups have emerged. Among these, the most prominent are: diphenylamine-methyl-piperazine, benzothiazole and benzoaxazole derivatives (Ciba-Geigy), ivermectins (Merck Sharp & Dohme), and benzimidazole carbamates (Janssen Pharmaceutica) (Godwin, 1984).

Many medicinal compounds now in use were originally found by accident. For example, suramin revealed its effectiveness against *O volvulus* when used for treatment of sleeping sickness in patients also suffering from onchocerciasis. In addition, the interest in the benzimidazole ring system was established in the 1950s, when it was found that BZ was an integral part of the structure of vitamin B_{12}. As a result of this interest, extensive studies have been made on BZ, and one health related area that has benefited greatly has been the treatment of parasitic diseases with BZ (Townsend & Wise, 1990). The more or less random selection of compounds must sooner or later lose impetus and, for advances to occur, a positive effort must be made to break new ground on a more rational basis. The logical design of antifilarial

drugs has, as yet, little basic molecular and biochemical information to work on. Knowledge of tubulins of filarial parasites would be extremely helpful in designing more potent and specific antimicrotubule agents. In order to understand the basis of this agents we must now consider the biology of the microtubules.

1.4. BIOLOGY OF MICROTUBULES

Microtubules (Fig. 1.9) are universal components of all the eukaryotic cells. They are found in an array of morphologically distinct structures and have been implicated in a wide range of motility-associated processes, including chromosome separation, intracellular transport of organelles, and maintenance of cell shape (reviewed in Roberts & Hyams, 1979; McIntosh, 1982; Dustin, 1984). They provide the cell with a dynamic structural framework with which it may mould various morphogenetic changes, and with an apparatus for the movement of organelles from place to place within the cellular environment. In both these functions, microtubules sometimes appear to integrate with intermediate filaments and microfilaments (Olmsted & Borisy, 1973; Andreu & Timasheff, 1986). The basic building block of the microtubule is the protein tubulin, which is a 6S dimeric 100,000 molecular weight protein composed of two non-identical ~50,000 molecular weight protein chain designated α and ß (Brayan & Wilson, 1971; Ponstingl *et al.*, 1981). The outside surface of microtubules can carry a wide variety of microtubule-associated proteins (MAPs) which seem largely responsible for the functional diversity of microtubules (Table 1.4) (Lightenberg *et al.*, 1988). Dynein is a MAP of special importance because its interaction with tubulin is essential for the motility of cilia and flagella (Gibbons, 1981; Mitchell & Rosenbaum, 1985).

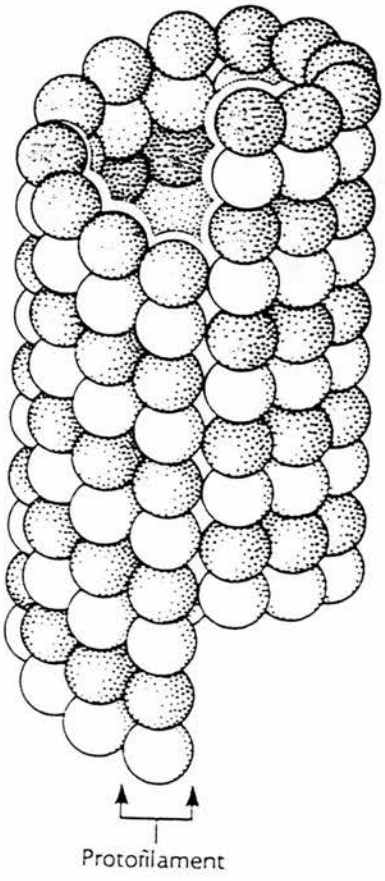


Fig. 1.9 Arrangement of tubulin molecules in a microtubule. Each sphere represents one tubulin molecule of about 50 kD (white, α; grey, β-tubulin). A longitudinal string of alternating α- and β-tubulin molecules is called a protofilament (width about 5 nm). The hollow microtubule cylinder is usually made up of 13 protofilaments. The functional building block is not a single tubulin molecule, but a combination of α- and β-tubulin (heterodimer, height 2 × 4 nm = 8 nm). The heterodimers are oriented along the protofilaments, i.e. the protofilaments consist of heterodimers joined head-to-tail. Thus the structure is polar; one end has a crown of α-tubulin, the other one of β-tubulin. Adjacent protofilaments are slightly shifted (by 1 nm) relative to one another so that corresponding subunits are nearly in register; this generates a set of shallow helices. However, symmetry arguments require that the helical symmetry is broken at least once. This helical discontinuity ('seam') is shown here between two central protofilaments where two unequal subunits are roughly in register. Although the protofilaments in this diagram are straight they are also capable of twisting around the microtubule axis ('supertwist').

TABLE 1.4. Microtubule-Associated Proteins

Group	Subunit mol. wt.(kD)	Comments
MAP-1	Three isoforms ~350	Isolated from brain, projected from microtubular wall; promotes assembly
Proteins associated with MAP-1 (light chains of MAP-1)	30 28	Associated with MAP-1 at stoichiometry 1 mole of each per mole of high-molecular-weight polypeptide
MAP-2	Two closely related isoforms ~270	Isolated from brain, projected from microtubular wall; promotes assembly
Proteins associated with MAP-2	70 54 + 39	Function unknown Subunits of cAMP-dependent protein kinase
Tau	Family containing several isoforms 55-62	Isolated from brain; promotes assembly
MAPs isolated from cultured cells of human HeLa line	Triplet centred at ~210; 125	Promotes assembly
MAP-3	180	Present in axons and glial cells
MAP-4	Triplet of related proteins 215-240	Isolated from mouse neuroblastoma cells occurred in different mouse tissues

Reviews: Vallee & Bloom (1984); Vallee *et al.* (1984); Vallee (1984). For HeLa MAPS: Bulinski & Borisy (1979). For tau proteins: Cleveland *et al.* (1977). For MAP-3: Huber *et al.* (1986). For MAP-4: Parysek *et al.* (1984).

Microtubules are polar structures with unequal ends, called plus and minus ends. These ends have a number of different properties; in particular, the growth of microtubules in tubulin solution is usually faster at the plus ends than at the minus ends (Bergen & Borisy, 1983). One or both ends of cellular microtubules are often associated with special structures called microtubule-organizing centres (MTOCs). Only a few types of MTOCs, namely centrosomes, basal bodies, and kinetochores, have been isolated from cells, and

biochemical studies have just begun (Osborn & Weber, 1976).

Microtubules exist in dynamic equilibrium with tubulin, the ratio of dimeric tubulin to polymeric microtubules being controlled by a range of endogenous regulatory proteins and co-factors (Fig. 1.10) (Margolis & Wilson, 1981; Lacey, 1988). This equilibrium can be altered, both *in vivo* and *in vitro*, by exogenous substances known as microtubule inhibitors. Most, but not all, such inhibitors exert their action by binding to tubulin to prevent the self-association of subunits onto the growing microtubules. This results in a 'capping' of the microtubule at the associating end while the microtubule continues to dissociate from the opposing end, with a net loss of microtubule length. One implication of this phenomenon is that it is not necessary for inhibitors to bind all tubulin dimers to inhibit polymerization, it is sufficient for them simply to 'cap' the microtubule (Lacey, 1988).

The effects of specific inhibitors on polymerization of tubulins deserve a special comment, since they are widely used in the study of microtubules.

1.4.1. Microtubule Inhibitors

Microtubule inhibitors are a group of structurally diverse compounds produced by fungi, plants, marine organisms, possibly higher eukaryotic animals and more recently, synthetically. They show a wide spectrum of selective and non-selective toxicity against eukaryotic genera.

Many groups of drugs are able to bind to tubulin and affect its polymerization both *in vitro* and *in vivo*.

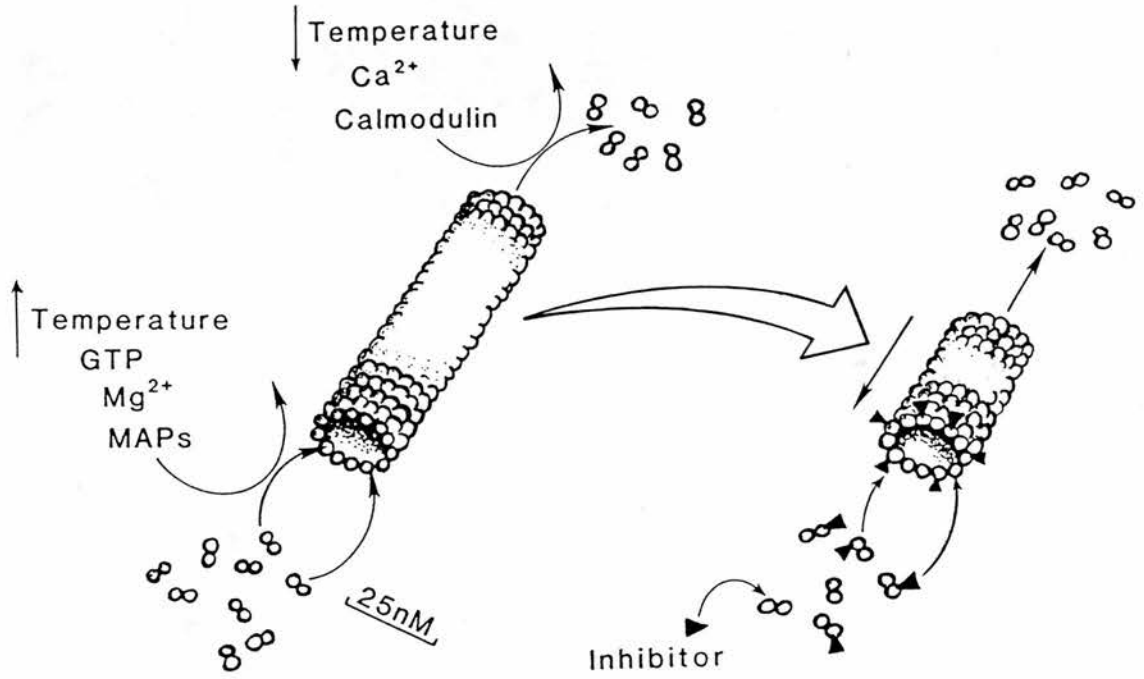


Fig. 1.10. Typical microtubules reach a dynamic equilibrium with tubulin, controlled by endogenous concentrations of co-factors such as GTP, Mg^{2+}, microtubule-associated proteins (MAPS), Ca^{2+} and calmodulin. Regulation in vitro, by increasing temperatures, enhances polymerization, whereas reducing the temperature induces depolymerization. Addition of inhibitors causes 'capping' and halts polymerization, allowing only depolymerization with the loss of microtubule structure.

These drugs are widely used in the study of microtubules. The best studied tubulin-specific drug is Colchicine, which is isolated from the plants *Colchicum autumnale* or *Colchicum speciosum*. This drug inhibits the polymerization of tubulin *in vitro* and induces fast disassembly of most types of cellular microtubules *in vivo* (Margolis & Wilson, 1977). One tubulin dimer can bind one colchicine molecule with high affinity. Colchicine is capable of inhibiting microtubule polymerization, and the concentrations required for this are considerably below the concentration of free tubulin molecules. For this reason, the phenomenon has been named "substoichiometric poisoning" (Margolis & Wilson, 1977; Williams *et al.*, 1985; Bergen & Borisy, 1983). It has been shown that in the vast majority of ß-tubulins the Cys residues at aa positions 239 and 354 are well conserved and involved in the binding of colchicine (Little & Luduena, 1986). In the *P. falciparum* (Wesseling *et al.*, 1989), S. cerevisiae (Kilmartin, 1981), and Arabidopsis thaliana *(Oppenheimer* et al., 1988) ß-tubulins the Cys_{239} is replaced by serine . Since the assembly of yeast tubulins is inhibited at higher concentrations of colchicine than that of mammalian tubulins (Kilmartin, 1981), it is likely that this also holds true for the other tubulins suggesting an altered sensitivity of those ß-tubulin for colchicine.

Several other drugs, such as colcemid, podophyllotoxin, and nocodazole, bind to the same site of the tubulin molecule as colchicine. Their effects on tubulin polymerization are also qualitatively similar to those of colchicine.

Vinblastine and vincristine form another group of drugs which are able to inhibit microtubule polymerization. These drugs bind to other sites of the tubulin molecule than colchicine (Wilson *et al.*, 1975), but they also substoichiometrically inhibit polymerization

of tubulin (Jordan *et al.*, 1986). Although most of the tubulin-specific drugs are too toxic for therapeutic use, vinblastine and vincristine have found a use in cancer chemotherapy (Hoebeke *et al*, 1976).

The possible physiological role of high-affinity binding sites for colchicine and vinblastine on the tubulin molecule constitutes an intriguing problem related to the study of microtubule-specific drugs. It may be suggested that these drugs are toxic analogues of some normal regulatory substances binding to the same sites and controlling assembly-disassembly of microtubules. But no molecules, playing a physiological role similar to colchicine or vinblastine, have been found in animal tissues (Borisy *et al.*, 1984).

Another interesting problem is related to the mechanisms controlling the sensitivity of cellular microtubules to specific drugs. In contrast to the microtubules of most other structures, the full-grown ciliary microtubules are resistant to colchicine. In usual conditions, cytoplasmic microtubules are highly sensitive to colchicine. But cytoplasmic microtubules become resistant to colchicine when the level of ATP in the cell is reversibly decreased by metabolic inhibitors. The sensitivity possibly depends on the spectrum of MAPs present on the microtubule. ATP deprivation can alter the state of these MAPs by inhibiting their phosphorylation (Boehm *et al.*, 1984; Lindwall & Cole, 1984; Murphy *et al.*, 1977).

The lack of adequate *in vitro* culture techniques for all stages of the helminth life cycle and for producing inbred strains of worms has hampered the investigations of benzimidazole action at the molecular level. However, the recent development of new techniques has opened this field to further research (Lacey, 1988).

Molecular studies of BZ resistance in eukaryotes indicate that it is caused by structural changes in the ß-tubulin gene. Binding studies of BZ to tubulin protein fractions of *Haemonchus contortus* showed that the resistant population had reduced binding affinity (Lacey & Prichard, 1986). This implied that there were structural changes in the ß-tubulin molecule (Duwel, 1987). The organization and location of the point mutations (Fig. 1.12) that result in amino acid changes in the ß-tubulin molecule have been identified in *C. elegans* (Driscoll, *et al.*, 1989), *N. crassa* (Orbach *et al.*, 1986), and in *S.cerevisiae* (Thomas *et al.*, 1985; Huffaker *et al.*, 1988; Matsuzaki *et al.*, 1988).

None of the mutations is located at any of the known functional sites (Fig. 1.12). Nevertheless functional differences have been found in *S. cerevisiae* where the mutations have not only resulted in resistance or hypersensitivity to BZs, but also to the thermosensitivity of the mutants. Such mutations may therefore influence the assembly of microtubules when the yeast is grown at different temperatures. In fact selection for thermosensitivity also produced ß-tubulin mutants with variable BZ-binding properties (Thomas *et al.*, 1985; Huffaker *et al.*, 1988). The point mutations will be considered in more detail in chapter 3.

The key to the understanding of these processes is likely to reside in both the tubulin subunits that assemble to form microtubules and the non-tubulin "associated" proteins (MAPs) that influence and mediate microtubule function. Thus, analysis of the molecular genetics of tubulin will provide important information to improve our knowledge of these processes.

1.5. TUBULIN

Tubulin is the main building block of all the microtubular systems. In the native form it is a dimer of two different polypeptides present in equimolar quantities, α- and ß-tubulin (Ponstingl *et al.*, 1981). The α-tubulin (pI 5.3) is slightly more basic than ß-tubulin (pI 5.1). These two subunits have about 40% of identical residues, and ß-tubulin is somewhat shorter than α-tubulin (~445/~451 residues) (Cleveland *et al.*, 1980). Most of the eukaryotic organisms analyzed so far carry more than one copy of both α- and ß-tubulin genes (Cleveland *et al.*, 1980; Wilde *et al.*, 1982). One exception is in *Toxoplasma gondii* where the α- and ß-tubulin genes are present as single copies in an haploid genome.

The carboxy-terminal 15-amino acid residues of ß-tubulin genes are characteristically diversified, whereas the rest of the amino acid sequence is highly conserved (Fulton & Simpson, 1976; Kowit & Fulton, 1974).

Owing to the conservation of tubulin structure, microtubules of very different organisms can often be detected by the same antibody (Helm *et al., 1989).* However, some exceptional results have also been reported. Thus, only minimal similarities were found between the tubulin gene of the protist *Naegleria* (Lai *et al.*, 1984) and chicken ß-tubulin gene (Sullivan *et al.*, 1984). This finding indicates that the tubulin structure may have undergone considerable changes in certain branches of the evolutionary tree.

Some 23 years ago, tubulin was originally identified as the soluble protein found in most eukaryotic cells that tightly bound the antimitotic drug colchicine (Wilson, 1966; Borisy & Taylor, 1967). Quickly thereafter,

this same protein was shown to be a major component of ciliary microtubules (Mohri, 1968; Shelanski & Taylor, 1968) and the principal protein subunit of microtubules in the cell cytoplasm (Weisenberg *et al.*, 1968).

These observations coupled with the later discovery of conditions that supported in vitro assembly and disassembly of tubulin into microtubules (Weisenberg, 1972) opened the way for a careful analysis of the tubulin polypeptide, its polymerization properties (Purich & Kristoferson, 1984; Dustin, 1984; Kirschner & Mitchison, 1986; McKiethan & Rosenbaum, 1984; Purich & Kristofferson, 1984), and its companion microtubule associated proteins (MAPS) (Olmsted, 1986).

Tubulin has not yet been crystallized, and therefore its structure is not known at atomic resolution. Several earlier studies have dealt with polymorphic assembly forms of tubulin that are amenable to electron microscopy and image processing. These works have yielded different modes of packing of the protein subunits, and their gross shape at about 2nm resolution, but no substantial progress has been made in this direction. Intact microtubules have also been studied by X-ray fibre diffraction and have led to a low resolution (2.5nm) structure of the hydrated particle. This approach has now been extended to 1.8nm resolution (Beese *et al.*, 1987). This showed that the microtubule wall is highly corrugated, and that tubulin may contain up to three structural domains. The earlier X-ray studies used microtubules repolymerized *in vitro* and thus left open the question of whether such a structure differed from the native one. However, Wais-Steider *et al.* (1988) showed that X-ray patterns from intact cytoplasmic microtubules are very similar, confirming that native and reassembled microtubules have comparable structures.

A wealth of data is now available on primary structures of various tubulins. Higher eukaryotes contain multiple genes for α- and ß- tubulin (Kemphues *et al.*, 1982), which generate isotypic variants of the protein (Cleveland, 1987). For instance, recent analysis revealed six isoforms of α-tubulin and 12 isoforms of ß-tubulin in brain tissue (Cleveland & Sullivan, 1985). Some of these isoforms are likely to be coded by different genes. The chick genome has four genes for each α- and ß-tubulin (Cleveland & Sullivan, 1985). Obviously, the number of isoforms in this case is larger than the number of genes. This suggests that post-translational modifications can generate at least a part of the tubulin heterogeneity (Gundersen *et al.*, 1984). In fact, several types of these modifications have been revealed in various organisms: tyrosination (Gunderson *et al.*, 1984; Geuens *et al.*, 1986), acetylation (Piperno *et al.*, 1987), and phosphorylation (Serrano *et al.*, 1987). Detyrosination and tyrosination are unique modifications of tubulin; no other protein is known to undergo these modifications (Kreis, 1987). The α-Tubulin polypeptide is synthesized with a tyrosine at its C-terminus. This tyrosine can be removed by a specific carboxypeptidase. This nontyrosinated α-tubulin is a substrate for addition of tyrosine by another specific enzyme, tubulin tyrosine ligase . Recently it was found that various microtubules of the same cultured cell contain either tyrosinated or nontyrosinated tubulin (Gundersen *et al.*, 1984). These results suggest that tyrosination-detyrosination can be used for the formation of functionally different groups of microtubules, the nature of which is not known. α-Tubulin present in the flagella of *Chlamydomonas* appears to be an acetylated form of the α-tubulin present in the body of this unicellular organism (Silflow *et al.*, 1985; L'Hernault & Rosenbaum, 1985). This is the only case in which specific post-translational modification of tubulin seems to be correlated with the special function of microtubules.

Interestingly, the isotypic variation is well-conserved among mammals and avians including mouse, human, and chicken (Wang *et al.*, 1986; Sullivan *et al.*, 1986; Cleveland, 1987).

The analysis of the human tubulin gene families has shown that many, if not most, of the human tubulin sequences are pseudogenes which contain multiple deletions and/or in frame translation termination codons within the exon sequences (Gwo-Shu Lee *et al.*, 1983; Wilde *et al.*, 1982). The nature of these tubulin pseudogenes is an intriguing story in itself. Only one of the nine human ß sequences is a 'traditional' pseudogene that contains intervening sequences. The other eight are of a novel and unexpected class characterized by (a) the lack of all intervening sequences, (b) the presence of a long coded poly-A tract at the downstream 3' end, and (c) the presence of a 10-15 base pair direct repeat in both the upstream and downstream flanking genomic DNA. These findings clearly imply that these pseudogenes arose by a reverse transcription event in which a mature tubulin mRNA was copied into DNA and inserted at a staggered chromosomal break (Wilde *et al.*, 1982). This class of pseudogene has also been seen in rat α tubulin (Lemischka & Sharp, 1982) and will probably prove to be a common eukaryotic phenomenon.

The study of tubulin regulation has also emerged as an important model system in which to dissect the fundamental mechanisms of eukaryotic gene regulation. Cells regulate tubulin gene expression at several levels: a) selection of which of multiple genes to express; b) coordination of the synthesis of α and ß tubulins; and c) control of the level of tubulin expression during the cell cycle and during development and differentiation. Regulation both at the level of mRNA stability (Yen *et al.*, 1988) as well as at the level of transcription has

been found to contribute to the proper orchestration of tubulin synthesis (Brunke *et al.*, 1982).

We have not endeavoured in the present to provide an exhaustive citation of the complete literature in tubulin, but rather attempted to discuss some points of interest for a better analysis of a ß-tubulin gene from *O gibsoni*, a filarial worm. These points include: microtubule genetics in *Drosophila*; autoregulated control of tubulin synthesis in animal cells; properties of ß-tubulin isotype classes; GTP-binding site; and MAPs binding site.

1.5.1. Microtubule Genetics in *Drosophila*

With the discovery in *Drosophila* of a dominant ß-tubulin mutation whose phenotype was male sterility, it was possible to investigate in detail the role of differential gene expression in construction of microtubules destined for different cellular functions. Kemphues *et al.* (1979; 1980) identified in *Drosophila* a ß-tubulin subunit, named ß2, which was expressed only in testis and was the sole ß-tubulin component of mature motile sperm. Such mutant was defective not only in axonemal assembly, but also in meiosis, suggesting that the ß2 subunit is multifunctional. This hypothesis was later confirmed by Kemphues *et al.*, 1982; 1983; Raff, 1984. In these works, two classes of recessive mutations in the ß2 locus were obtained. The first class, distinguished by production of a ß2 polypeptide that is unstable, yields a greatly reduced pool of both α- and ß-tubulin subunits and a complete blockage of meiosis, nuclear shaping, and axenomal assembly in mutant spermatocytes (Kemphues *et al.*, 1982; 1983; Raff & Fuller, 1984). The second class, characterized by a stable ß2 subunit, consists of a set of mutants each with a distinct phenotypic pattern of microtubule disruption (Raff, 1984;

Raff & Fuller, 1984). Since by definition, a recessive mutation does not disrupt the wild type function, it seems clear that the recessive ß2 polypeptides do not interfere with the wild-type subunits and that ß2 subunits are a normal component in the various sperm microtubules.

1.5.2. Autoregulated Control of Tubulin Synthesis in Animal Cells

A priori it seems obvious that eukaryotic cells can and do regulate their tubulin content. Indeed, such regulation could be achieved either at the level of polymer or the level of the α/ß subunit or both. In a pioneering experiment which addressed this question, Ben-Ze'ev *et al.* (1979) proposed that cultured mammalian cells modulate tubulin gene expression through a feedback control mechanism linked to the level of unpolymerized tubulin subunits. These results emerged from analyzing the level of tubulin synthesis in cells treated with drugs that interfere with normal microtubule assembly. For example, following colchicine-induced microtubule depolymerization (and a corresponding increase in the level of unpolymerized subunits) there is a specific repression of new tubulin polypeptide synthesis (Weber *et al.*, 1975). Treatment of cells with a second microtubule inhibitor, vinblastine (Hiller & Weber, 1978; Spiegelman *et al.*, 1979) (which not only induces microtubule depolymerization but also leads to precipitation of the unpolymerized subunits into paracrystals), led to a very different result: tubulin synthesis was specifically elevated. Thus it was proposed that regulation of tubulin expression was autoregulated by the level of unassembled subunits. This initial work was quickly confirmed by Cleveland *et al.* (1983) by directly elevating tubulin subunits inside cells, using microinjection. With this approach, a rapid and specific suppression of new tubulin synthesis was achieved. Overall, it was shown that

inhibitors that increase the concentration of unassembled subunits (colchicine and nocodazole) induced suppression of a new synthesis, whereas those that lower the intracellular subunit level (vinblastine and taxol) stimulated the level of the new synthesis. This apparent autoregulation of the synthesis has been demonstrated in almost all animal cells tested (Cleveland *et al.*, 1981), including those from species as divergent as mosquito and human (Cleveland & Havercroft, 1983). The original report by Ben Ze'ev *et al.* (1979) also demonstrated that the changes in tubulin synthesis were reflected in corresponding changes in translatable tubulin mRNA levels.

Once sequences for α- and ß-tubulin were cloned, it was relatively simple, using RNA blotting, to show that the primary effect was on tubulin mRNA levels (Cleveland *et al.*, 1981). Thus, recent works (Gay *et al.*, 1987; Yen *et al.*, 1988), using transient DNA transfection of hybrid gene constructs carrying portions of tubulin gene, have identified the sequences carried by ß-tubulin RNA that label it as a substrate for selective degradation when the tubulin subunit concentration is elevated. It was thus clear that the domain that could confer autoregulated instability was a 13-nucleotide segment that encoded the amino-terminal four amino acids of ß-tubulin (Yen *et al.*, 1988). Finally, this 13-nucleotide domain proved not only sufficient for autoregulation but also necessary, since deletion of codon 2 or codon 3 and 4 from an otherwise authentic ß-tubulin gene disrupted autoregulated instability of its RNA transcripts (Yen *et al.*, 1988). On the basis of these findings, Yen *et al.* (1988) have proposed a model for autoregulated instability of ß-tubulin mRNA, where unpolymerized tubulin subunits bind directly (or activate a factor which binds) to the nascent amino-terminal tetrapeptide (MREI) of ß-tubulin. This binding is transduced through the adjacent ribosome to activate an RNAse which degrades the ribosome-bound mRNA.

This model is shown in Fig. 1.11.

1.5.3. Properties of ß-Tubulin Isotype Classes

The diversity of microtubule function, coupled with the demonstration of differential stability of different microtubule classes to depolymerization induced by drugs or other agents (Behnke & Forer, 1967; Brinkley & Cartwright, 1975), fuelled speculation that different microtubule classes might be assembled from different tubulin subunits, thereby establishing functionally distinct microtubules (Osborn & Weber, 1976). This has turned into the long-standing question of whether different tubulin isotypes serve different functions. Although this may be true in some cases (e.g. the microtubules of the marginal band of avian erythrocytes (Cleveland, 1987)), several recent studies show that different tubulins copolymerize indiscriminately (Lopata & Cleveland, 1987; Gu *et al.*, 1988). Thus, the role of tubulin diversity remains open. However, the conservation of structure and regulatory functions among ß-tubulin genes in different species allowed the identification of six major classes of ß-tubulin polypeptide isotypes (Sullivan & Cleveland 1986; Wang *et al.*, 1986; Lopata & Cleveland, 1987; Monteiro & Cleveland 1988). The C-terminal sequence of each of the vertebrate ß-tubulin isotypes (classes I-VI) determined in chicken and mammals are shown in Table 1.5.

The expression of isotypes classes of ß-tubulin is both complex and highly conserved (see Table 1.5; Farmer *et al.*, 1984; 1986; Havercroft & Cleveland, 1984; Griffin *et al.*, 1985; Lewis *et al.*, 1985; Wang *et al.*, 1986; Ginzburg *et al.*, 1985; Denoulet *et al.*, 1986). The class I gene encodes an abundant, ubiquitously expressed isotype, while class II represents a major neural ß-tubulin isotype associated with neural differentiation and

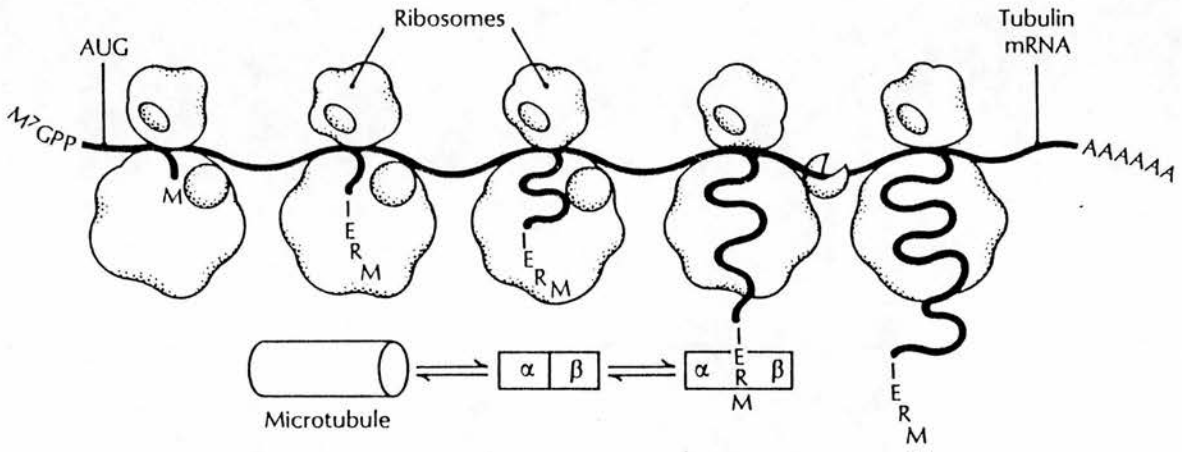


Fig. 1.11. Proposed model for autoregulated instability of ß-tubulin messenger RNA (mRNA). Unpolymerized tubulin subunits bind directly [or activate a factor(s) which binds] to the nascent amino terminal tetrapeptide (Met-Arg-Glu-Ile) of ß-tubulin. This binding is transduced through the adjacent ribosome to activate an RNAse which degrades the ribosome-bound mRNA. The RNAse has been drawn (❍) as ribosome-associated, although this has not yet been demonstrated. MREI is the amino terminal ß-tubulin polypeptide.

regeneration (Hoffman *et al.*,1988). Class III is a minor neural polypeptide, apparently specific to chordates (Little & Luduena, 1985; Sullivan & Cleveland, 1984). The class IVa gene is expressed abundantly in neural tissue in later stages of development in the rat and the mouse, while the closely related class IVb gene is expressed as the major testes ß-tubulin as well as a minor component of a variety of tissues. Class V gene expression has only been examined in chicken, where it was found in all tissues and cell types examined except for neurons (Sullivan & Cleveland, 1986). Finally, the class VI isotype is expressed only in cells of the haematopoietic lineage, where it comprises the major ß-tubulin of marginal band microtubules (Murphy *et al.*, 1986; Lewis *et al.*, 1987). Only the class III, IVa, and VI genes show simple pattern of tissue specific expression. The remaining isotypes are coexpressed in variable quantities in many cell types, and all cells appear to express multiple isotypes.

Two groups of sequences can be discerned among the 6 ß-tubulin isotypes. The sequences of isotypic classes I, II, and IV are closely related, differing by only 2-4%, whereas isotypes classes III, V, and VI are more divergent, differing at 8-16% of amino acid positions. The expression of class I, II, and IV isotypes is relatively abundant, while class III and V isotypes are less abundantly expressed. The haematopoietic class VI protein is the most abundant ß-tubulin in the differentiated cells in which it is found. While the divergent group isotypes show no obvious overall relatedness, they all share a Cys_{239}-Ser_{239} substitution, normally a conserved site, and except for a mammalian ß-tubulin gene, mß1, an Ala_{124}-Cys_{124} substitution. In chicken, where the expression of each isotype has been examined, all cell types express at least one divergent isotype, perhaps reflecting a requirement for representatives of

both groups of ß-tubulin isotypes in vertebrates cells (Sullivan *et al.*, 1986).

Table 1.5. Properties of the vertebrate ß-tubulin isotype classes*

Isotype	Gene/ protein	C-terminal isotype defining sequence	Expression
Class I	cß7 mß5 rbt. 3 hß1	EEEEDFGEEAEEEA	Constitutive; many tissues
Class II	cß1/cß2 mß2 rbt. 1 hß2 porc. ßA bov. ß1	DEQGEFEEEGEEDEA EG	Major neuronal; many tissues
Class III	cß4 hß4 porc. ßB bov. ß2	EEEGEMYEDDEEESEQGAK EEEGEMYEDDEEESESQGPK	Minor neuronal; neuron specific
Class IVa	mß4 rbt. 2 h5ß	EEGEFEEEAEEEVA	Major neural; brain specific
Class IVb	cß3 mß3 hß2	EEEGEFEEEAEEEAE EEEGEFEEEAEEEVA	Major testes; many tissues
Class V	cß5	NDGEEEAFEDDEEEINE	Minor constitutive; absent from neurons
Class VI	cß6 mß1	DVEEYEEAEASPEKET GLEDSEEDAEEAEVEAEDKDH	Major erythrocyte/platelets; specific

* The source of the sequences are: Class I, cß7 (Monteiro & Cleveland 1988); mß5 (Lewis *et al.*, 1985); rbt.2 and rbt.3 (Farmer *et al.*, 1984); hß1 (Hall *et al.*, 1983). Class II, cß1 and cß2 (Valenzuela *et al.*, 1981; mß2 (Lewis *et al.*, 1985); rbt.1 (Farmer *et al.*, 1984); hß2 (Cowan *et al.*, 1981); porc.ßA (Krauhs *et al.*, 1981); bov.ß1 (Little & Luduena 1985). Class III cß4 (Sullivan & Cleveland 1984); hß4 (Sullivan & Cleveland,1986); porc.ßB (Krauhs *et al.*, 1981); bov.ß2 (Little & Luduena, 1985). Class IVa, mß4 (Lewis *et al.*, 1985); rbt.2 (Farmer *et al.*, 1984); h5ß (Gwo-Shu Lee et al., 1984). Class IVb, cß3 (Sullivan *et al.*, 1986); mß3 (Wang *et al.*, 1986). Class V, cß5 (Sullivan *et al.*, 1986). Class VI, cß6 (Murphy *et al.*, 1987); mß1 (Wang et al., 1986).

1.5.4. GTP-Binding Site of ß-Tubulin

The tubulin dimer is known to bind to one exchangeable and one nonexchangeable GTP (GTPe and GTPn, Jacobs *et al.*, 1974). A significant portion of tubulin's folding pathway is determined by the need to accommodate and bind one molecule of GTP for each subunit. GTP bound to the ß-chain is exchangeable whereas the GTP bound to the α-chain is non-exchangeable (Geahlen & Haley, 1977; Nath *et al.*, 1985; Hesse *et al.*, 1985). The GTPe is hydrolysed to GDP during polymerization. But the GTPn is firmly bound to the dimer and does not participate in the polymerization reaction. The role of the GTPn is still unknown (Jacobs *et al.*, 1974). Consequently, the resulting microtubule contains one molecule of GDP and one molecule of GTP for each dimer, and the binding and hydrolysis of GTPe regulates microtubule assembly.

Sequence similarities have been reported among GTP-binding proteins and both α- and ß-tubulin protein (Wierenga & Hol, 1983; Sternlicht *et al.*, 1987; Halliday, 1984; Leberman & Egner, 1984). Comparing α- and ß-tubulin to other nucleotide-binding proteins, it appears that there are four regions of similarities -- termed I, II, III, and IV in Fig. 1.12. All regions I-IV are highly conserved in the sequences of ß-tubulin known thus far (Cleveland & Sullivan, 1985); moreover, they are conserved between α- and ß-tubulin. This would be expected if these regions had an important function such as GTP binding. Region I (at position 140-146 -- GGGTGSG) is the glycine cluster that probably represents the turn between a ß-sheet and an α-helix as part of a nucleotide-binding motif (Schulz & Schirmer, 1979) which is involved in the binding of phosphates. Region II (at position 177-182 --TVVEP) is thought to represent the end of another ß-strand that could contribute to the binding of the ribose, whereas Region III (at position 239-244 --LRFPG), is thought to be

near the base of the nucleotide, as judged by homology with ATP- and dinucleotide binding proteins (e.g. dehydrogenases pp60). However, there is another region that is also thought to be near the base in a class of GTP-binding proteins, represented for example by EF-Tu or p21, which is called Region IV (at position 63-77 -- AILVDLEPGTMDSVR), the binding site of GTPe recently confirmed by Linse & Mandelkov (1988), using direct photoaffinity labelling. Despite some earlier ambiguities, there appears now to be a consensus that the binding site of the exchangeable GTPe is on ß-tubulin (Geahlen & Haley, 1977; Hesse *et al.*, 1987), and, more specifically, on its N-terminal domain defined by chymotryptic cleavage (Nath & Himes, 1986; Linse & Mandelkov, 1986). Such site was located within the peptide consisting of residues 63-77, AILVDLEPGTMDSVR a highly conserved sequence in ß-tubulin molecule (Fig. 1.12). The mutations represented in Fig. 1.12 will be considered in chapter 3.

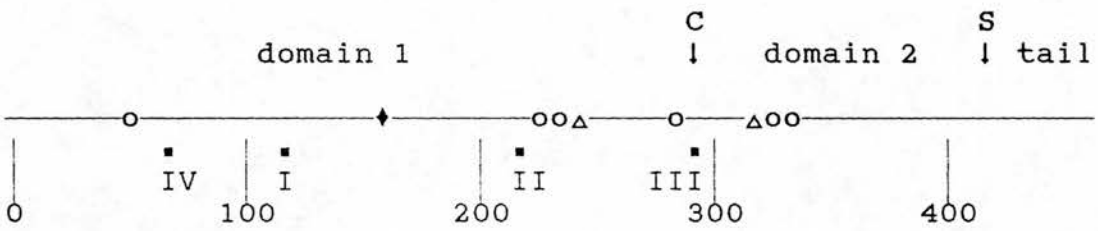


Fig. 1.12. Characteristics of the ß-tubulin molecule. ß-tubulin can be cleaved into specific fragments by chymotrypsin (C) and subtilisin (S) resulting in domain 1 (34kDa), domain 2 (19kDa), and a tail fragment of 2kDa (Sacket & Wolff, 1986; Mandelkov *et al.*, 1985). The region around the chymotrypsin cleavage site, the hinge region between the two domains, facilitates the assembly of tubulin and the growth of microtubules. Deletion of the tail increases the capacity for tubulin polymerization. Mutations in the tail region probably confer functional specificity to isotubulins (Serrano *et al.*, 1988). ß-tubulin is able to bind one molecule of GTP; comparison with other GTP-binding proteins predicts that several sites (I, II, III, and IV) are involved in binding, probably acting together in the three dimensional structure (Sternlicht *et al.*, 1987; Linse & Mandelkov, 1988). Symbols: o, mutations in *C. elegans* (Driscoll *et al.*, 1990); ♦, mutations in *N. crassa* (Orbach *et al.*, 1986); ▵ mutations in *S. cerevisiae* (Thomas *et al.*, 1985; Huffaker *et al.*, 1988).

1.5.5. Microtubule-Associated Proteins (MAPs) Binding Sites

The functional diversity of microtubules along with their intracellular dynamics suggest the existence of several regulatory signals, at the level of microtubule-associated proteins (MAPs) or tubulin, which could finely control microtubule assembly and orientation in the cell (Soifer, 1986; Maccioni, 1986). Macromolecular interactions between microtubular components is an aspect crucial to understanding the functions and regulation of microtubule assembly. Recently, Serrano *et al.* (1984b) have demonstrated that a 4kd tubulin fragment, including amino acids residues from Phe_{418} to Glu_{450} in α-subunit and Phe_{408} -Ala_{445} of the ß-sequence, plays a major role in controlling tubulin interactions leading to microtubule assembly. The 4kd carboxyl-terminal domain also constitutes an essential domain for the interaction of microtubule-associated proteins (Serrano *et al.*, 1984a; Maccioni *et al.*, 1985; Sackett & Wolff, 1986).

Elegant studies using a biological approach based on direct binding measurements of synthetic peptides to MAPs (Maccioni *et al.*, 1988), together with observations from both immunologic and limited proteolysis studies, indicate that: 1) the subdomain including residues 430-441 in α-tubulin and 422-434 in ß-tubulin plays an essential role in the selective interaction with MAPs; 2) the location of those domains is in the outer surface of the microtubule; 3) it is possible that regions adjacent to this domain may contribute directly or indirectly to the productive interaction of MAPs with tubulin and that other short discrete sequence could be implicated in the binding of a large macromolecule like MAP-2; 4) there is a relatively strong interaction of MAP-2 with the ß(422-434) peptide and a weaker interaction of both MAPs components with α(430-441) tubulin peptide; 5)

multiple sites for the binding of the peptides in both MAP-2 and tau are present; 6) the binding of ß(422-434) peptide to tau has a co-operative nature. All the evidence presented above indicates that those interactions must be biologically meaningful.

Tubulin has now been studied for 23 years, and what has became abundantly clear is that the systems of microtubules seem to be regulated by a hierarchy of various mechanisms. This system has a number of features which made it a particularly attractive candidate for investigation. Studies on tubulin of onchocerciasis infective agents, in particular *O. gibsoni*, which so little is yet known, would provide a substantial contribution to our understanding of gene organization and expression in the human filarial parasite. Any differences between the parasite and the host in the properties of these proteins can potentially be exploited in rational drug design strategies. For example, effectiveness of benzimidazole compounds as anthelmintic drugs appears to be due to their preferential action on nematode tubulin (Dawson *et al.*, 1989).

1.6. THE GENOME OF FILARIAL PARASITES

1.6.1. Molecular Karyotype.

The haploid chromosome number has been described from at least ten species of filarial nematode and the basic chromosome number appears to be five pairs (N = 5). This number was found in *Acanthocheilonema viteae* (Terry *et al.*,, 1961) *Dirofilaria immitis* (Sakaguchi *et al.*, 1980; Delves *et al.*, 1986; Taylor, 1960), *Wuchereria bancrofti* (Miller, 1966), *Brugia pahangi* and *B. malayi* (Sakaguchi *et al.*, 1983); Delves, 1986), and *Onchocerca gutturosa* (Hirai *et al.*, 1985; Delves, 1986).

Post *et al.* (1989) found the same number after examining ovaries and testes of species of *Onchocerca* (*O. ochengi, O. gutturosa, O. armillata* and *O. lienalis*), but in contrast they also found that *O. volvulus* and *O. gibsoni* had only four pairs. The karyotype of filarial nematodes usually consists of five pairs of chromosomes, and the apparent reduction to four pairs might have occurred independently in *O. volvulus* and *O. gibsoni*. However, this might also be the result of a single evolutionary event, which would indicate that *O. volvulus* is phylogenetically more closely related to *O. gibsoni* than to the other *Onchocerca* species examined (Post *et al.*, 1989).

1.6.2. Genome Size

The genome size is one of the fundamental characteristics that has to be known before embarking on molecular biological investigations of any organism. It is an important indicator of the size of the libraries that have to be maintained and the possible number of different recombinants that are to be screened.

The size of the *Onchocerca* parasite genomes have not been determined. However McReynolds *et al.* (1986) have found that the complexity of the genome of the filarial worm *B. malayi* is $8x10^7$. This is close to the published estimate of the size of the genome of the free-living soil nematode *C. elegans,* $8x10^7$ (Sulston & Brenner, 1974), and it is about one-fortieth of that of man.

1.6.3. Repetitive DNA.

Rothstein & Rajan have shown that the genome of these filarial parasites is composed of 10-20% repetitive DNA and 80-90% unique copy DNA (Rajan, 1990). The repetitive DNA families appear to fall into several

classes -- those whose sequence is specific for the species from which they are isolated, those whose sequence is shared between members of the genus, and, between these two extremes, members with varying degrees of overlap between the species that comprise the genus.

Considerable work has been done on the repetitive DNA of filarial nematodes. The genomes of *Brugia* and *Onchocerca* genera appear to contain at least one family of tandem repetitive DNA [named the *HhaI* family in *Brugia* (McReynolds *et al.*, 1986; Williams et al., 1988)], with monomer repeats in the order of 10^2 bp [322 bp in *Brugia* (McReynolds *et al.*, 1986) and 149 bp in *Onchocerca* (Meredith *et al.*, 1989; Murray et al., 1988)].

Most highly repeated DNA sequences do not appear to code for an active protein or RNA product. Thus, these sequences are free to evolve at a much faster rate than the coding sequence. Using this diversity, it is possible to generate probes specific for various species. Sensitivity levels capable of detecting a single organism in field quality samples have been achieved (Piessens *et al.*, 1987), and promise to have great impact on epidemiological studies and in diagnostic applications.

1.6.4. Base Composition.

The base composition of an organism is a valuable parameter in the analysis of its relationships with other organisms.

The percentage of guanine (G) plus cytosine (C) in duplex DNA differs in various organisms, ranging from 17-19% in the various malarial parasites (Pollack *et al.*, 1982) to about 72% in *Micrococcus* sp. (Marmur & Doty, 1962). The amount of G + C in DNA (referred to as GC

content) is an important, but by no means definite, criterion in determining evolutionary relationships. Thus, organisms that are closely related tend to have similar GC content while those that are more distantly related often differ. While the GC content cannot be used in isolation to define such relationships, it is one of several parameters that are useful in such analyses.

In order to obtain more information about the phylogenetic relationships of filarial organisms infecting different mammalian hosts, Rothstein *et al.* (1988) have determined the base composition of a number of filarial parasites, and of the free-living soil nematode *C. elegans*. The extremely low GC content of these organisms (26-28%) is significantly different from the free-living nematode (36-44%). These data imply that probes isolated from organisms of higher GC content cannot hybridize to the homologous genes from the filarial organisms. For instance, histone, myosin and actin probes from *C. elegans* used on genomic Southern blots of filarial organisms gave essentially no hybridization (Rothstein *et al.*, 1988).

1.6.5. Minor Bases.

An additional useful parameter of analysis for eukaryotic genomes is the presence of minor bases. Most higher organisms contain minor components which are usually the four major bases modified in one or more ways. Rothstein *et al.* (1988) analyzed *B. malayi* DNA for the presence of such bases by HPLC analysis following complete hydrolysis. Such analyses fail to reveal the presence of even small amounts of methylated bases. This fact is of some interest, since methylated bases are believed to play an important role in gene regulation in higher organisms, and their absence in *B. malayi* DNA raises interesting questions about the mechanisms by which such gene regulation may occur in filarial parasites.

1.7. A PROFILE OF CLONED GENES FROM *Onchocerca*

Much of the work in filarial parasites, as with other infectious agents, has been directed towards identifying and characterizing genes that might encode protective antigens (Nanduri & Kazura, 1989). An extremely successful strategy in characterising filarial antigens, and their genes, as in the case of other parasites, has been to immunoscreen cDNA expression libraries with antibody probes. Probes have included human immune sera, monoclonal antibodies, animal antisera directed against *Onchocerca*, as well as taking advantage of cross-reacting antisera specific for a particular protein from a different filarial parasite. This strategy has also been complemented by utilising heterologous gene probes from other filarial parasites to screen libraries.

By this approach a number of genes encoding for major immunogen have been cloned and sequenced. These include heat-shock protein, myosin, paramyosin, major sperm protein.

1.7.1. 18kD Microfilarial Major Surface Antigen

As a step towards characterising parasite molecules strategically located at the host-parasite interface, Dinman & Scott (1990) have characterised an *O. volvulus* microfilarial antigen gene. Surface radio-iodination studies on *O. volvulus* microfilariae identified a prominent 18kD band. The strategy they employed to clone this component was to exploit the immunological cross reactivity which exists between a 16kD microfilarial surface component of the filarial nematode parasite of dogs, *Dirofilaria immitis*, and this 18kD molecule (see also Maizels *et al.*, 1985). The cross-reacting antibodies

were used to immunoscreen libraries, a clone designated M2f.e, was isolated and sequenced. M2f.e was found to contain an open reading frame of 495bp encoding an 18kD protein.

To confirm the identity of this clone, a 347bp restriction fragment was subcloned into a plasmid expression vector and an antiserum generated against the recombinant fusion protein. The antiserum recognised a 18kD component in extracts of *O. volvulus* microfilariae and bound to the surface of intact *O. volvulus* and *D. immitis* microfilariae in immunofluorescence experiments.

Southern 'zoo' blots demonstrated that M2f.e-like sequences occur in the DNAs of a number of filarial nematode species, but were not observed in non-filarial nematodes or mammalian organisms. This suggests that M2f.e-like sequences have been conserved during filarial evolution and may encode for proteins that are critical for parasite survival.

1.7.2. 42kD Infective Larvae Stage Antigen

Antigens from the infectious form of the filarial parasite, the third stage larva or L_3, are of immunoprophylactic potential as it has been demonstrated in *D. immitis* and *B. malayi* that vaccination with irradiated infectious larvae can induce protection (Wong *et al.*, 1974; Yates & Higashi, 1985). However *O. volvulus* infective larvae must be isolated by dissection of infected blackflies which are difficult to obtain. To circumvent the problem of the lack of *O. volvulus* material from the L_3 state for analysis, Unnasch and coworkers (1988) adopted a rather unusual approach. These workers have utilised an antiserum produced by introducing living infective larvae into a non-permissive host (a rabbit) to immunoscreen an *O. volvulus* cDNA expression library. As

the *O. volvulus* library was constructed from adult mRNA this would allow only the isolation of antigen genes expressed in both stages of the life-cycle.

A clone was isolated from this library designated λRAL-1 with an insert of 1076bp. The insert had a single ORF of 1008bp immediately from the 5'-end of the fragment followed presumably by a 3' untranslated region. The ORF encodes for a protein of a predicted size of at least 39kD. The 1076bp insert hybridises to a single message in adult *O. volvulus* mRNA of 1450 bases and as the ORF of clone λRAL-1 contains no initialling methionine codon it appears that a 5' portion of the coding region is missing from the λRAL-1 clone. Furthermore, antibodies immuno-affinity purified from the antisera on induced cultures of λRAL-1 lysogens recognise a single 42kD band in western blots of adult worm extracts.

Curiously, the ORF encodes three repeats of the heptapeptide KKPEDWD. Repeated peptide sequences have also been observed in other parasite antigens in particular in *Plasmodium*, the causative agent of malaria (Enea *et al.*, 1984). The recombinant antigen produced by the λRAL-1 clone stimulated T-cells from infected individuals to proliferate. As the cellular immune response may be important in the development of immunity against helminthic infections, this antigen may have immunoprophylactic value (Lal *et al.*, 1987).

1.7.3. 33kD Reproductive Organ and Muscle Antigen

A 33kD immunodominant antigen has been characterised and the corresponding gene partially cloned and sequenced by Lucius *et al.* (1988a, 1988b). The antigen is of immunodiagnostic potential as it is recognised by 96% of sera from onchocerciasis patients but not from patients infected with other filarial parasites.

A monoclonal antibody directed against the antigen in immunofluorescence assays revealed that the antigen is present in reproductive organs and muscles but not in the cuticle. A polyclonal antiserum directed against the immunoaffinity purified antigen was used to immunoscreen a cDNA expression library. Four positive clones were isolated with different sized inserts all of which cross-hybridised, suggesting that they encode the same sequence. Clone OV33-3 contained the largest EcoRI cDNA insert of 1kb in size. This clone produced a recombinant fusion protein which is also recognised by the monoclonal antibody and human patient sera. DNA sequence analysis of this clone revealed it to encode for 239 amino-acids in frame with the ß-galactosidase gene of λgt11, followed by a 3' untranslated region of 254 bases but missing the poly A tail. Northern blot analysis identified a band of 1200 bases in size suggesting that the OV33-3 cDNA lacks approximately 200 nucleotides of the mature message.

The nucleotide and amino-acid sequence of OV33-3 was compared to those in GenBank and revealed no significant similarities. To determine whether OV33-3-like sequences are present in other filarial parasites, Southern blot analysis of the genomic DNA under reduced stringency conditions of hybridisation revealed OV33-3-like sequences are also present in the filarial parasites *D. immitis* and *B. malayi* despite the lack of immunological cross-reactivity. However even under low stringency the cDNA probe did not hybridise to genomic DNA from the free living nematode *C. elegans* suggesting that OV33-3-like sequences appear to be confined to parasitic nematodes.

1.7.4. Heat Shock Protein-Like Antigen

Human infected with filarial parasites produce a variety of clinical symptoms. To investigate the

possibility that members of different clinical groups respond to different filarial antigens, Rothstein *et al.* (1989) immunoscreened *O. volvulus* expression libraries with sera from a number of individuals belonging to different clinical groups. Their studies identified a number of cloned antigens which were differentially recognised according to clinical symptoms. The insert from a clone that was primarily recognised by amicrofilaremic hosts was sequenced. The 966bp insert contained a single open reading frame which displayed remarkable similarity to the *Xenopus laevis* heat shock protein 70 (HSP 70) and the rat heat shock cognate protein (Bienz, 1984; O'Malley *et al.*, 1985). Surprisingly, the N-terminal 285aa were highly conserved (92% identity) with *X. laevis* HSP 70, but the remaining 36aa showed considerable divergence (65% identity).

This study supports the original data of Selkirk *et al.* (1987), who adopted a similar approach to define antigens differentially recognised by filarial patients. These workers screened *Brugia pahangi* libraries with patient sera and isolated a clone primarily recognised by amicrofilaremic individuals. This clone too encoded a HSP 70-like protein which has 98% sequence identity with the *O. volvulus* protein.

The 'heat shock' family of genes contains members which are inducible by stresses other than heat and some which are constitutively expressed. These genes may play a vital role in the developmental regulation of parasitic organisms.

1.7.5. Major Sperm Protein

Nematode spermatozoa are non-flagellated crawling cells (Anya, 1976). They contain an abundant protein, the major sperm protein (MSP), which represents 15% of the total protein and is presumed to function as a

cytoskeletal element involved in motility (Nelson *et al.*, 1982).

Scott *et al.* (1989) have utilised an *Ascaris* MSP cDNA fragment as an heterologous probe to screen *O. volvulus* λgt11 genomic libraries. Two different genomic clones termed OVGS-1 and OVGS-2 with insert sizes of 765bp and 1765bp were isolated and characterised. Sequence analysis revealed that both the genomic clones contain MSP coding regions of 99 and 282bp separated by an 153bp intron. Over the coding regions OVGS-1 and OVGS-2 are only 95% similar and similarity drops sharply to only 79% in their intron sequences. Introns will be discussed in more detail in chapter 3. Thus it appears that there are at least two MSP genes in the genome of *O. volvulus*, a copy number similar to that reported for other filarial parasites including *Ascaris* (Bennet & Ward, 1986). However in the free-living nematode *C. elegans* there exists an even more complex MSP gene family with more than 50 members including pseudogenes (Ward *et al.*, 1988).

The nucleotide sequences of the *O. volvulus* are over 80% similar to *Ascaris* MSP cDNA sequences and 79% similar to the *C. elegans* MSP-3 cDNA. The predicted amino acid sequences are even more closely related and the *O. volvulus* MSP genes are 90% similar to the *Ascaris* MSP and 82% similar to the *C. elegans* MSP-3 amino acid sequence.

Hence the results suggest that the MSP gene and its protein products have been highly conserved during nematode evolution. As the sperm cells from *Ascaris* and *Caenorhabditis* lack significant amounts of actin, myosin, and tubulin, the MSP's are the most likely candidates for the cytoskeletal protein required for motility. Furthermore, the MSP's present themselves as potential chemotherapeutic targets in the control of filariasis.

1.7.6. 200kD Myosin-Like Protein

Donelson and colleagues (Donelson *et al.*, 1988; Erondu & Donelson, 1990) have immunoscreened unamplified *O. volvulus* cDNA expression libraries with patient sera or rabbit antisera directed against a lysate of *O. volvulus*. Sixteen double positive clones were isolated, seven of which crosshybridised with each other, suggesting they encoded the same gene. The sequence of a 2kb cDNA insert from one of the crosshybridising clones, designated Onchoag-1, was determined. It contained a single open reading frame with an unusual stretch of 78 nucleotides rich in A bases encoding for a run of lysine residues. The authors felt that this unusual feature merited further investigation which subsequently revealed the 3' 78 nucleotides to be a cloning artifact. The actual sequence for this region was obtained by applying PCR to template cDNA extracted from an aliquot of the total λgt11 cDNA library and by utilising a primer 5' to this unusual sequence with the λt11 reverse primer. A prominent band of 300bp was amplified and its sequence determined. It contained a single open reading frame the first 66 nucleotides of which matched the corresponding region of Onchoag-1 but the remainder of the sequence was unrelated to the unusual 3' 78 bases originally obtained from the Onchoag-1 cDNA.

To verify that this new sequence actually does represent the 3' end of the Onchoag-1 mRNA, genomic clones were isolated from a λEMBL library. Sequence determinations of PCR amplification products from these clones and also total genomic DNA not only confirmed the presence of this new 3' sequence in the Onchoag-1 chromosomal gene but also demonstrated the presence of several introns. Within a 1135bp segment of the cDNA gene analyzed seven introns were observed in the chromosomal gene ranging in size form 100 to 1200bp. As already

mentioned, the introns will be discussed in further detail in chapter 3.

Analysis of the deduced amino acid sequence of Onchoag-1 has 43% similarity with the *C. elegans* myosin heavy chain taking into consideration conservative changes. The secondary structure predicted by the conformational parameters of Chou & Fassman (1978) suggests that the Onchoag-1 protein has a high potential for an α-helix conformation with a segment having the capacity to form α-helical coiled-coils found in some structural proteins such as myosin.

The Onchoag-1 cDNA was subcloned into a pGEX expression vector and the resultant glutathione transferase fusion protein was purified and polyclonal antibodies generated. In western blots of adult female *O. volvulus* extracts the antisera recognised a 200kD band. Furthermore, the antisera specifically labelled muscle tissues in indirect immunofluorescent microscopy in sections of adult male and female O. *volvuli*. Thus these studies add more convincing evidence to suggest that Onchoag-1 is indeed a myosin-like myofibrillar protein.

1.7.7. Paramyosin Protein

Paramyosin is a protein which interacts with myosin and is found in the thick filaments of invertebrate muscle (Cohen *et al.*, 1971). The protein is also an important immunogen in several filarial infections and may be of immunoprophylactic value (Grandea *et al.*, 1989; Pearce *et al.*, 1986).

The gene encoding paramyosin has been cloned and sequenced in a number of organisms including *S. mansoni, C. elegans,* and *D. immitis,* but only the *C. elegans* gene sequence is complete (Kagawa *et al.*, 1989; Lanar *et al.*,

1986). By taking advantage of a crossreacting antiserum raised against recombinant *D. immitis* paramyosin Limberger & McReynolds (1990) immunoscreened an *O. volvulus* cDNA expression library. A number of clones have been isolated and approximately half of the gene sequenced.

A comparison of the nucleotide and amino acid sequences of the worm paramyosin genes reveals a number of interesting features. The nucleotide sequences of the *O. volvulus* and *C. elegans* genes are very close to that of *D. immitis* (94% and 70% respectively) but the *S. mansoni* nucleotide sequence is only distantly related to the *D. immitis* gene (49%). The similarities and differences between the paramyosins become more pronounced when amino acid sequences are taken into consideration. The *O. volvulus* protein shares 94% amino acid identity and the *C. elegans* protein shares 70% identity to the *D. immitis* DNA sequence. In contrast, the protein sequence of *S. mansoni* paramyosin has only 34% similarity to *D. immitis* paramyosin. However, most of the amino acid differences involve conservative changes and if similarly charged amino acids are also taken into account, then the similarity rises to 64%.

1.7.8 Ribosomal Gene.

B. malayi probably possesses more than one class of ribosomal genes that are tandemly repeated (Shah *et al.*, 1986; McReynold *et al.*, 1986; Sim *et al.*, 1986).

Sim *et al.* (1987) studies on *B. malayi, B. pahangi* and *D. immitis* reveal that the 4.3 kb coding region for 28S ribosomal RNA is separated from the 1.9 kb coding region for 18S rRNA by a 1 kb spacer region at the 3' end by a spacer region of about 500 bp at the 5' end. Previous studies of the rDNA of related *Xenopus* species revealed that, although the structural genes coding for 18S and 28S rRNAs are identical, the non-transcribed spacer sequences differ considerably (Forsheit *et al.*, 1974). Such observations suggest that spacer sequences evolve more rapidly than rRNA coding sequences. However, subsequent studies with the ribosomal genes of *Drosophila* showed that in this genus the non-transcribed spacer region is highly conserved among related species during evolution (Tartof, 1979). The attempt to determine whether such relationships exist in *B. malayi, B. pahangi* and *D. immitis*, shows that the non-transcribed spacer region of rRNA genes are conserved among those filariids (Sim *et al.*, 1987). Their function, however, remains unknown.

The availability of this rapidly enlarging data base of information promises to shed light on the vexing question of phylogenetic relationships among the various filarial parasites and their precise position in the evolutionary history of the phylum Nematoda.

Analysis of the limited rDNA sequence currently available has already permitted some insights into the relationships between the species of the genus *Onchocerca* (Gill *et al.*, 1988).

1.8. THE FOCUS AND AIMS OF THIS STUDY

Filarial parasites are just beginning to be studied at the genetic level. The potential of recombinant DNA technology for identifying parasite genes that are important in the pathogenesis of filarial disease or for the survival of the parasite is enormous. Work in several laboratories has already identified genes encoding ribosomal RNAs, as well as highly repeated DNA sequences that can be used as diagnostic probes. In addition, new methods to separate chromosomes will allow the physical mapping of parasite genes without the requirement for classical genetic analysis, which would be difficult in filariids.

So little is yet known about those parasites, that the adoption of more practical criteria to select a gene for initial study is convenient. In this spirit, it is reasonable to assume that an adequate approach should be not only feasible within the context of the small amount of information available so far, but also capable of providing the significant data which are needed in order to improve our knowledge of filarial worms.

The study of tubulin seems to be an adequate choice. For microtubules are essential organelles of eukaryotic cells serving a critical role in cellular physiology. They are composed of α- and ß-tubulin subunits which over vast evolutionary distances are among the most highly conserved proteins. The aim of this thesis is to clone the ß-tubulin gene from *O. gibsoni* by taking advantage of this high sequence conservation and to utilise a ß-tubulin gene probe from a different organism.

The cloning and subsequent sequencing of the ß-tubulin gene from *O. gibsoni* should provide an insight into the organisation and chromosomal structure of an

Onchocerca gene. As unlike a number of other *Onchocerca* genes cloned and partially sequenced to date this gene is present in other distant organisms allowing an evolutionary comparison to be drawn.

Furthermore, there is strong evidence to suggest that the effectiveness of anthelmintic drugs appears to be due to their preferential action on nematode tubulin. Thus, comparing and contrasting the ß-tubulin gene from *Onchocerca* spp with that of their mammalian hosts may help to highlight other chemotherapeutically exploitable differences.

CHAPTER 2

MATERIALS AND METHODS

2.1. BIOLOGICAL MATERIALS

2.1.1. Bacterial Strains, Bacteriophages and Vectors

All *Escherichia coli* strains, bacteriophages, and vectors used in this study are listed below with their genotypes.

***E. coli* strain**	**Genotype and reference**
NM514	*lyc7, hfl*, lyc7, $hsdR^{-}M^{+}S^{+}$ (Murray, 1983)
NM522	*hsd*Δ($M^{-}S^{-}R^{-}$) Δ*lac*, Δ*pro*, *sup*E *thi*/F' *pro*$A^{+}B^{+}$, *lac*I^{q}, *lac*Z, ΔM15, *tra*D36 (Gough & Murray 1983)
JM83	*ara*, Δ(*lac-pro*A<B), *rps*L, η80, *lac*Z, ΔM15 (Vieira & Messing, 1982)
TG_1	K12, Δ*lac-pro*, supE, *thi*, *hsd*D5, F' *tra*D36, *pro*$A^{+}B^{+}$ *lac*I^{q} *lac*Z ΔM15
ED8654	SupE, supF, hsdR, trpR, lacY (Borck *et al.*, 1976)

Bacteriophage	**Genotype and reference**
NM1149	λ Imm^{434}, b(538) (Murray, N. E. 1983)

Vectors	**Reference**
M13mp18,mp19	Yanisch-Perron *et al.* (1985)

PUBS	Murphy, G (personal communication)

2.1.2. Media and Solutions

Antibiotics	L-Broth or molten L-Agar was supplemented with an appropriate antibiotic when necessary; ampicillin was added to 100μg/ml, kanamycin to 430μg/ml, and tetracycline to 10μg/ml.
BBL Agar	Baltimore Biological Laboratories trypticase, 10g; NaCl, 5g; Difco agar, 10g; per litre (pH unaltered).
BBL Top Agar	As for BBL Agar, but only 6.5g Difco agar per litre.
Homogenisation Solution (HS)	10mM tris-HCl (pH 7.4); 10mM EDTA; 60mM NaCl; 0.15mM spermine; 0.15mM spermidine; 0.5% (v/v) triton x-100 (sterilized and kept cold at 4°C).
Minimal Medium	Difco Bacto Agar, 15g; 100ml Spizizen salts (5x); 10ml 20% (w/v) glucose; 0.125ml 5mg/ml Vitamin B1; per litre.

L Agar	Difco Bacto Tryptone, 10g; Difco Bacto yeast extract, 5g; NaCl, 10g; Difco agar, 15g; per litre adjusted to pH 7.2.
L Broth	Difco Bacto Tryptone, 10g; Difcto Bacto yeast extract, 5g; NaCl, 5g; per litre adjusted to pH 7.2.
PBS	NaCl, 8.76g; $Na_2HPO_4.2H_2O$, 17.8g; $NaH_2PO_4.2H_2O$, 7.8g; per litre (pH 7.2).
Phage Buffer	KH_2HPO_4, 3g; Na_2HPO_4 (anhydrous), 7g; NaCl, 5g; 1mM $MgSO_4$; 0.1mM $CaCl_2$; 1ml of 1% Gelatin solution per litre.
REact Buffer 1	50mM Tris-HCl (pH 8.0); 10mM $MgCl_2$.
REact Buffer 2	50mM Tris-HCl (pH 8.0); 10mM $MgCl_2$; 50mM NaCl.
REact Buffer 3	50mM Tris-HCl (pH 8.0); 10mM $MgCl_2$; 100mM NaCl.
REact Buffer 4	20mM Tris-HCl (pH 7.4); 5mM $MgCl_2$; 50mM KCl.
REact Buffer 5	10mM Tris-HCl (pH 8.2); 8mM $MgCl_2$.
REact Buffer 6	50mM Tris-HCl (pH 7.4); 6mM $MgCl_2$; 50mM KCl; 50mM NaCl.

REact Buffer 7	50mM Tris-HCl (pH 7.4); 10mM $MgCl_2$; 50mM KCl; 50mM NaCl.
REact Buffer 8	20mM Tris-HCl (pH 7.4); 10mM $MgCl_2$.
REact Buffer 9	50mM Tris-HCl (pH 8.5); 5mM $MgCl_2$.
REact Buffer 10	100mM Tris-HCl (pH 7.6); 10mM $MgCl_2$; 150mM NaCl.
REact Buffer 11	10mM Tris-HCl (pH 9.0); 12mM $MgCl_2$; 100mM KCl.
(5x) Spizizen Salts	$(NH_4)_2SO_4$, 2g; K_2HPO_4, 6g; trisodium citrate, 1g; $MgSO_4$, 0.2g; per litre.
STE	10mM tris-HCl (pH 7.4); 1mM EDTA (pH 8.0); 0.1M NaCl.
X-Gal Indicator	Molten L-Agar, supplemented with an appropriate antibiotic if necessary, is poured into a plate and allowed to solidify. After drying at 37°C, 200µl of L-Broth, containing 30µl og X-Gal (20mg/ml, 5-bromo-4-chloro-3-indoyl ß galactosidase in dimethylformamide), and 20µl of IPTG (100mM, isopropyl-ß-D-thiogalactoside in double distilled water), is spread onto the plate.
TE (pH 7.6)	10mM Tris-HCl (pH 7.6); 1mM EDTA

(pH 8.0).

TAE (50 X) 250g Tris base; 57.1ml glacial acetic acid; 100ml 0.5M EDTA pH 8.0.

TBE (5 X) 54g Tris base; 27.5g boric acid; 20ml 0.5M EDTA pH 8.0.

20 X SSC 175,3g NaCl, 27.6g *di*-Sodium hydrogen citrate and 7.4g EDTA were dissolved in 800ml H_2O; the pH was adjusted to 7.4 with NaOH 10N; the volume was adjusted to 1l with H_2O; the mixture was sterilized by autoclaving.

2.1.3. Preparation of Plating Cells

A single bacterial colony was grown to saturation overnight at 37°C in 10ml of L Broth supplemented with 10mM $MgSO_4$ and 0.1% (w/v) maltose, shaking at 200 cycles/minute.

The overnight culture was diluted 1/50 in 100ml of supplemented L Broth and incubated with aeration at 37 °C until O D_{600} = 0.3-0.5. The cells were harvested by centrifugation (1000g; 5 min) and resuspended in 10mM $MgSO_4$ to an appropriate density (Abs_{600} = 2, i.e., approximately $1.6x10^9$ cells/ml) which corresponds to a dilution of approximately 1/10 (10ml) of the original culture volume. The cell stock was stored at 4°C for up to one week.

2.2. NUCLEIC ACID METHODS

2.2.1. Phenol/Chloroform extraction of Nucleic Acids

The phenol required was redistilled, adjusted to 0.1% (w/v) 8-hydroxyquinoline and saturated with TE. After purification phenol was stored at -20°C till required.

The DNA extraction was as follows.
An equal volume of phenol was added to DNA in TE. The mixture was incubated at 37°C for 5 mins and occasionally vortexed to maintain the emulsion. After cooling on ice, an equal volume of $CHCl_3$/isoamyl alcohol (24:1) was added. The resulting mixture was incubated for 5 mins vortexing occasionally. Phases were separated by centrifugation and the aqueous phase was removed. The organic phase was back-extracted with an equal volume of TE. The aqueous phases were combined and then re-extracted with an equal volume of $CHCl_3$/isoamyl alcohol (24:1) on ice. After centrifugation, the aqueous phases were precipitated as required.

2.2.2. Precipitation of Nucleic Acids

The aqueous phase was adjusted to 0.3M sodium acetate (pH 5.2) or 0.4M NaCl. 2 volumes of ethanol or 1 volume of isopropanol was added. The mixture was incubated at -20°C for at least 1 hr. The precipitate was recovered by centrifugation at 4°C in a Sorvall centrifuge (10,000rpm, 20 mins, HB4) or microfuge (12,000rpm, 10 mins). The pellet was washed in 70% ethanol, air dried and redissolved in ddH_2O or TE. Sometimes, DNA was also precipitated by the addition of an equal volume of 4M ammomium acetate and 2 volumes of ethanol or 1 volume of isopropanol. After incubating on liquid N_2 for 5 mins, the DNA was recovered as above. Two such precipitations could remove above 99% of unincorporated nucleotides from

reactions.

2.2.3. Quantification of Nucleic Acids

In order to quantify nucleic acids, the optical density at 260nm was used. An OD_{260} of 1.0 is 50μg/ml for DNA or 40μg/ml for RNA. DNA was free of contaminating protein if OD_{260}/OD_{280} was 1.8–2.0. The background value at OD_{320} was subtracted from all readings. A rough estimate of nucleic acid concentration could be obtained by ethidium bromide staining of agarose gels with known quantities of material.

2.2.4. Restriction Enzyme Digestion of DNA

Restriction enzymes were mainly obtained from Bethesda Research Laboratory (BRL). Restriction digests of DNA were performed in accordance with the suppliers instructions (Table 2.1), usually at 37°C with two units of enzyme per μg of DNA for 1–5 hrs (Table 2.2). Digestions were completed by the addition of EDTA pH 8.1 to a final concentration of 10mM and heating at 65°C for 10 mins. Whenever the buffering requirements of each enzyme permitted, multiple digestions were performed simultaneously. Otherwise, those enzymes requiring lower salt conditions were added before the salt, and pH was subsequently changed.

Table 2.1. Restriction Digestion Conditions.
(From BRL)

Enzyme	REact Buffer	Activity of enzyme in each of the four basic Buffers			
		1	2	3	4
Acc I	1	**100**	<10	*	100#
Alu I	1	**100**	67	40	80
Apa I	4	25	<10	<10	**100**
Ava I	2	83	**100**	25	125#
Ava II	2	50	**100**	20	100#
Bal I	9	30	<10	<10	20
BamH I	3	75	125#	**100**	40
Bcl I	2	140#	**100**	60	50
Bgl I	2	20	**100**	100#	40
Bgl II	3	100#	100#	**100**	50
BstE II	2	75	**100**	75	100#
Cfo I	1	**100**	20	<10	90
Cla I	1	**100**	100#	125#	125#
Cvn I	4	40	<10	<10	**100**
Dde I	2	30	**100**	50	20
Dpn I	4	67	100#	67	**100**
Dra I	1	**100**	70	70	135#
EcoR I	3	*	120#	**100**	80
EcoR II	6	<10	<10	<10	<10
EcoR V	2	25	**100**	125#	30
Hae II	2	125#	**100**	67	125#
Hae III	2	75	**100**	80	125#
Hha I	2	75	**100**	50	30
Hinc II	4	<10	50	50	**100**
Hind III	2	30	**100**	50	80
Hinf I	2	100#	**100**	50	80
Hpa I	4	30	50	10	**100**
Hpa II	8	67	10	*	60
Kpn I	4	60	<10	<10	**100**
Mbo I	2	120#	**100**	60	50

continued

Table 2.1. Continuation

Enzyme	REact Buffer	Activity of enzyme in each of the four basic Buffers 1	2	3	4
Mbo II	1	**100**	70	30	85
Mlu I	3	25	50	**100**	15
Msp I	1	**100**	25	25	30
Nar I	1	**100**	30	20	<10
Nci I	8	<10	<10	<10	80
Nco I	3	100#	133#	**100**	60
Nde I	2	10	**100**	100#	15
Nde II	10	50	75	133#	10
Nru I	7	<10	<10	67	15
Nsi I	3	50	125#	**100**	20
Pst I	2	35	**100**	50	50
Pvu I	7	20	33	60	10
Pvu II	6	30	50	30	40
Rsa I	1	**100**	50	10	80
Sal I	3	<10	30	**100**	<10
Sau3A I	4	<10	<10	<10	**100**
Sau96 I	11	20	10	<10	<10
Sca I	6	*	*	*	<10
Sma I	4	<10	<10	<10	**100**
Sph I	6	150#	30	40	40
Sst I	2	150#	**100**	50	90
Sst II	2	135#	**100**	<10	70
Stu I	2	120#	**100**	50	100#
Sty I	3	50	100#	**100**	<10
Taq I	2	50	**100**	75	50
Tha I	1	**100**	67	<10	100#
Xba I	2	60	**100**	30	125#
Xho I	2	50	**100**	100#	50
Xma III	5	50	<10	<10	20
Xor II	4	30	<10	<10	**100**

All Buffers were stored at -20 °C

Notes:

For a number of enzymes, activity values are ≥100% in buffers that are not recommended for use by BRL. In these cases, a higher than acceptable level of contaminants, such as nonspecific endonuclease activity, has been observed. These enzymes and buffers should *not* be used together.

* Use of this enzyme in this REact Buffer causes significant 'star' activity and is *not* recommended.

Table 2.2. Restriction Endonuclease Stability.
(From BRL)

Restrict. Endonucleases	Hours* Complete Activity	Hours# Partial Activity	Restrict. Endonucleases	Hours* Complete Activity	Hours# Partial Activity
Acc I	5	N.D.	*Mbo* II	3	5
Alu I	5	N.D.	*Mlu* I	5	N.D.
Apa I	5	N.D.	*Msp* I	1	2
Ava I	5	N.D.	*Nar* I	5	N.D.
Ava II	3	5	*Nci* I	5	N.D.
Bal I	1	5	*Nco* I	3	5
*Bam*H I	1	2	*Nde* I	1	5
Bgl I	1	5	*Nru* I	1	5
Bgl II	1	5	*Nsi* I	1	2
*Bst*E	1	5	*Pst* I	1	2
Cfo I	1	1	*Pvu* I	3	5
Cla I	5	N.D.	*Pvu* II	1	4
Cvn I	1	2	*Rsa* I	1	2
Dde I	1	5	*Sal* I	2	5
Dpn I	1	5	*Sau* 3A I	1	2
Dra I	1	3	*Sau*96 I	5	N.D.
*Eco*R I	5	N.D.	*Sca* I	1	1
*Eco*R II	1	2	*Sma* I	1	4
*Eco*R V	1	4	*Sph* I	1	1
Hae II	1	5	*Sst* I	5	N.D.
Hae III	2	5	*Sst* I	5	N.D.
Hha I	1	4	*Stu* I	5	N.D.
Hinc II	1	5	*Sty* I	1	1
Hind III	2	5	*Taq* I	3	5
Hinf I	1	3	*Tha* I	1	2
Hpa I	1	5	*Xba* I	2	5
Hpa II	5	N.D.	*Xho* I	5	N.D.
Kpn I	1	2	*Xma* III	5	N.D.
Mbo I	1	5	*Xor* II	1	5

N.D. Not determined. Restriction endonucleases that showed 5 hour stability were not tested in the presence of sheared salmon sperm DNA.
* The values refer to the total period of time the enzyme was incubated at the reaction temperature.
# The values were determined in the presence of sheared salmon sperm DNA.

2.2.5 Extraction of Parasite DNA

Frozen parasites were quickly ground to powder in the presence of liquid Nitrogen and transferred to a homogeniser containing 10ml of ice-cold homogenisation solution. The homogenate was spun down at 6.000g 7 min at 4°C and the pellet washed for 3 times. Lysis occurred in homogenisation solution containing 2% (w/v) of sarkosyl and 100 mg/ml proteinase K for 1 h at 50°C, swirling periodically. After RNAase treatment, phenol/chloroform extraction was performed as in section 2.2.1.

The DNA was not precipitated with ethanol. Instead, traces of chloroform were removed by dialysing the DNA solution extensively against large volumes of ice-cold STE.

2.2.6 Agarose Gel Electrophoresis

2.2.6.1 Large Agarose Gels

DNA was fractionated in conformity with molecular weight by electrophoresis. 0.6-2% gels were used. Electrophoresis was performed through horizontal gel slabs measuring 28 x 14 x 0.5cm; wicks connected both ends of the gel with the electrophoresis buffer tanks. The composition of the buffer was 50mM Tris-HCl, 10mM tri-sodium citrate, 1mM EDTA; with the help of glacial acetic acid, pH was adjusted to 8.2. Later on, the buffer was supplemented with ethidium bromide (500µg/l). The agarose gel type II (Sigma) was melted in the same buffer. Before electrophoresis, a loading buffer (10% Ficoll; 0.025% bromophenol blue) was added to 10% of the sample volume.

Samples were run into the gel at 10V/cm, and subsequently overnight at 4V/cm.

2.2.6.2. Mini Agarose Gels

Small amounts of DNA (0.05μg-0.5μg) were fractionated by using mini agarose gels measuring 10 x 5 x 0.15cm.The gel and electrophoresis buffer was composed of Tris-borate (89mM Tris-HCl pH 8.2; 89mM Boric Acid, 25mM EDTA) and ethidium bromide (500μg/l). Electrophoresis was carried out at 15V/cm for 30-60 mins.

2.2.7. DNA Molecular Weight Markers

The DNA molecular weight markers employed were either a 123 bp ladder purchased from Bethesda Research Laboratories, with fragments ranged by size from 123 bp to 4182 bp, or phage λcI857 DNA restricted with *Hind*III, with 23.1kb, 9.4kb, 6.6kb, 4.4kb, 2.3kb, 2.0kb, and 0.56kb sized fragments.

2.2.8. Gel Photography

DNA was visualised in stained gels by means of an ultraviolet transilluminator (254nm). Photographs were taken using a red (A1) filter onto Ilford HP5 film (f4.5, 20 secs). Films were processed in Ilford microphen for 5 mins, stopped in 3% (v/v) acetic acid for 30 secs, and fixed in Ilford hyper for 5 mins at room temperature. Films were well washed in water and then dried. The relative mobilities of the sample were directly measured from the negative (distance was proportional to $\log_{10}$ mwt).

2.2.9. Autoradiography

This was performed using CRONEX 4 X-ray film in a cassette with lightning plus intensifying screens. The cassettes were stored at -70°C for the required time.

2.2.10. Ligation of DNA

With the appropriate restriction endonucleases, the vector and insert DNA were cut to completion. The resulting samples were phenol extracted (2.2.1), precipitated (2.2.2), and then dissolved in ddH_2O. Ligation conditions were chosen in a way such that the vector to insert DNA concentrations were at a 2:1 molar ratio. 100ng vector and 20ng insert DNA were ligated in 15μl reactions containing 1 x T4 DNA ligase buffer, 1mM ATP and 1 unit T4 DNA ligase at 15°C for 18 hrs. The ligation mixes were transformed into *E. coli* or *in vitro* packaged into lambda phage.

2.2.11. Transformation of *E. coli*

A single colony of *E. coli* was picked into 5ml L Broth and grown up overnight at 37°C. 1ml was extracted from the overnight culture and used to inoculate a 100ml culture which was grown till OD_{650} = 0.2. Cells were initially chilled on ice and next spun down. The cell pellet was gently resuspended in 50ml of cold 100mM $CaCl_2$ and then conserved on ice for 15 mins. Cells were respun and the pellet was resuspended in 10ml of cold 100mM $CaCl_2$ and conserved on ice for at least 1 hr before use.

To the DNA sample, which was less than 100ng, 0.2ml of competent cells were added in glass tubes and conserved on ice for 1 hr. The tubes were heat shocked for 2 mins at 42°C. 1ml L Broth was added and the mixture was incubated at 37°C for 1 hr. Molten BBL top agar (2.5ml at 45°C) was added. The resulting mixture was poured onto appropriate plates, inverted and incubated at the correct temperature. If lacZ colour selection was used, then 30μl X-gal and 30μl of IPTG were added before pouring. This method gave 10^5-10^7 transformants/μg DNA.

2.2.12. Extraction of DNA from Agarose Gels

2.2.12.1. Low Melting Point Agarose Gel (Weislander, 1979)

Low melting point agarose gels were run as indicated in section 2.2.6. The required DNA fragment was excised cleanly from the gel. In order to melt the gel, 5 vols of 0.1M NaCl, 10mM Tris-Cl pH 8.0, 1mM EDTA were added and heated to 65°C. The resulting mixture was phenol extracted (no $CHCl_3$) once at 65°C, and then the aqueous phase was phenol extracted as described in section 2.2.1. and precipitated as in section 2.2.2.

2.2.12.2. Electroelution

Pharmacia agarose gels were run as previously described (section 2.2.6). A small trough was cut in front of the desired fragment. The trough was lined with dialysis tubing, and the fragment was electrophoresed into the trough. The current was reversed for 30 secs and then the DNA was sucked off with a pasteur pipette. Next, the DNA was phenol extracted as described in section 2.2.1, and then precipitated as in section 2.2.2.

2.2.13. Elutip-d Column Purification of DNA

For some purposes, further purification of DNA was required. Elutip-d columns were used as described by the manufacturer (Shleicher and Schull): columns were primed by washing in high salt buffer (1.0M NaCl, 20mM Tris-Cl pH 7.5, 1mM EDTA), then low salt buffer (0.2M NaCl, 20mM Tris pH 7.5, 1mM EDTA); the columns were attached to 0.45μm cellulose acetate filters to remove particulate matter; DNA, in low salt buffer, was applied to the columns, then eluted in high salt buffer (no filter attached in this stage), and the sample was precipitated.

2.2.14. Plasmid DNA Preparation

2.2.14.1. Mini-preparation of Plasmid DNA

A single bacterial colony was used to inoculate 50ml culture in L Broth with adequate antibiotics and grown overnight by shaking at the required temperature (usually 37°C). The cells were collected by centrifugation for 15mins and the pellet resuspended on ice in 3.5ml of 25mM Tris-HCl pH 8.0, 10mM EDTA and 15% sucrose. After this, 10mg/ml of lysozyme were added and the incubation on ice was carried on for 30 mins. 8ml of fresh 0.2M NaOH 1% SDS were added, vortexed hard and left on ice for further 30 mins. After this, 5ml 3M NaOAc pH 5.2 were added, vortexed hard, left on ice for further 10 mins, and spun (15,000rpm, rotor SS34, 30 mins). The supernatant was collected, treated with 20μl RNAse (stock solution: 10mg/ml) at 37°C for 30 mins, phenol/chloroform/ isoamyl alcohol (25:24:1) extracted, and then precipitated with AmOAc and isopropanol. The pellet was resuspended in TE, treated with RNAase once again, and then phenol/chloroform extracted for three times until clear interface. It was then ethanol precipitated and resuspended in TE (200μl).

2.2.14.2. Maxi-preparation of Plasmid DNA

A 2ml culture of single bacterial colony was inoculated in L Broth with antibiotics and grown overnight usually at 37°C. Using 1ml of the overnight culture, a fresh 25ml culture was grown for 3 hrs. The 25ml culture was employed to start a 500ml culture. After 2 hrs, 75mg chloramphenicol was added and the culture grown overnight. Cells were harvested by centrifugation in a Sorvall GSA rotor (4,000rpm, 5 mins, 4°C), then resuspended in 5ml sucrose buffer (25% w/v sucrose, 50mM Tris-Cl pH 8.1, 40mM EDTA) on ice. 1ml 10mg/ml lysozyme (in sucrose buffer) and 1ml 0.5M EDTA were added. The mixture was incubated for 15

mins on ice. Lysis was obtained by the addition of 13ml Triton buffer (0.1% w/v Triton X-100, 10mM EDTA, 50mM Tris-Cl pH 8.0) and incubation on ice for a further 10 mins. Chromosomal DNA and cellular debris were removed under centrifugation in a Sorvall SS34 rotor (17,000rpm, 1 hr, 4°C). The supernatant was recovered, phenol/chloroform extracted as described in 2.2.1, and precipitated with ethanol (2.2.2).

DNA was recovered by centrifugation. It was then resuspended in TE containing 100μg/ml RNAse A, and incubated at 37°C for 30 mins followed by phenol/chloroform extraction and ethanol precipitation. The recovered plasmid DNA was resuspended in 3ml TE. 3g of caesium chloride and 0.15ml 5mg/ml ethidium bromide in TE were added. Exactly 6.15g were transferred to a vertical rotor centrifuge tube and plasmid DNA was banded by centrifugation in an OTD50 Sorvall ultracentrifuge (45,000rpm, 18°C, 18 hrs). The supercoiled plasmid DNA band was collected by means of a syringe needle pushed through the centrifuge tube wall. In order to remove ethidium bromide, plasmid DNA was extracted four times with butan-2-ol (saturated in 4 NaCl, TE), precipitated with isopropanol, resuspended in TE and precipitated once more with ammonium acetate. Finally, it was stored at -20°C in ddH_2O or TE.

2.2.15. Large-Scale Bacteriophage Lambda DNA Preparation

2.2.15.1. Plating of Phages

An overnight culture of NM514 (recombinant phage) was grown in L Broth supplemented with 0.4% (w/v) maltose, and collected by centrifugation. It was then resuspended in 1/2 volume of 10mM $MgSO_4$. Adequate dilutions of phage were mixed with 0.2ml of plating cells and then incubated at 37°C for 15 mins. 3ml of molten top agar

complemented with 10mM $MgSO_4$ was added. The mixture was then poured onto 9cm BBL agar plates. Set plates were inverted and incubated at 37°C for 6-12 hrs.

2.2.15.2. Plate Lysates

A well isolated phage plaque was picked into 1ml phage buffer. 50µl phage were incubated with 100µl plating cells and plated onto freshly poured L Plates. Phage were grown (non-inverted) at 37°C for 12 hrs, and then 4ml L Broth with 10mM $MgSO_4$ was added. The top layer and liquid were collected, vortexed briefly. Cells and agar were removed by centrifugation. The supernatant was collected, titred and a few drops of $CHCl_3$ were added. It was then stored at 4°C.

2.2.15.3. Liquid Lysates

A 500 ml culture of cells was grown at 37°C to OD_{650} = 0.5 in L Broth with 10mM $MgSO_4$. Cultures were infected with 1ml of plate lysate then incubated at 37°C with vigorous shaking until lysis occurred (usually 3-4 hrs). 10ml of chloroform were added, and the incubation was continued for further 10 mins at 37°C. After this, 500µg RNAse A and 500µg Dnase were added and the lysate was incubated at room temperature for 1 hr. 29.2g of NaCl were added, left for 1h at RT and the debris removed by centrifugation at 8,000rpm in Sorvall GSA rotor for 10 mins. Phages were precipitated overnight at 4°C by the addition of solid PEG-6000 to a final concentration of 10% w/v. The phage particles were recovered by centrifugation (Sorvall GSA rotor, 8,000rpm). The particles were gently resuspended in 5ml phage buffer.

2.2.15.4. Equilibrium Centrifugation in CsCl

The phage particles were then purified in CsCl in the following way. 0.75g of solid CsCl per mililiter of bacteriophage suspension were added and gently mixed in order to dissolve. The bacteriophage suspension was then transferred to an ultracentrifuge tube. The tube was filled with SM containing 0.75g/ml CsCl and was centrifuged for 24 hrs (Beckman Ti50 rotor, 35,000rpm, 4°C). The band of bacteriophage particles was collected and stored at 4°C in a tightly capped tube.

2.2.15.5. Phage DNA Extraction

The dialysed phage suspension was diluted to 2ml with TE. 100μl EDTA (0.5M), 100μl 10% (w/v) SDS and 10μl proteinase K (20mg/ml) were added. The mixture was incubated for 1 hr at 65°C. The phage DNA was then phenol extracted and precipitated with ethanol. The DNA pellet was left to redissolve in 500μl ddH_2O overnight at 4°C before storing at -20°C.

2.2.16. Small-Scale Bacteriophage Lambda DNA Preparation

The phage clone was plated at a dilution to give confluent lysis on five moist BBL agar plates and incubated overnight at 37°C. The top agar was scraped into phage buffer; next, it was removed by centrifugation (Sorvall GSA rotor, 5,000rpm, 10mins). In the presence of 0.2% maltose a 250ml culture of NM514 was grown to an OD650 = 0.4. It was then pelleted by centrifugation (Sorvall GSA rotor, 8,000rpm, 10 mins) and resuspended in 10ml of L Broth with 10mM $MgSO_4$. The cells were incubated with the phage at 37°C for 20 mins without shaking to allow adsorption. The mixture was diluted into 250ml of L Broth, with 0.2% maltose and 10mM $MgSO_4$, and was grown at 37°C till complete lysis had occurred. Cellular debris was

removed by centrifugation (Sorvall GSA rotor, 8,000rpm, 10 mins, 4°C). The phage were pelleted from the supernatant by centrifugation (Beckman Type 19 rotor, 18.500rpm, 3 hrs, 4°C).

The phage pellets were resuspended overnight in 10ml of phage buffer with gentle shaking at 4°C. Caesium chloride was added at 0.74g/ml in order to give a final density of 1.45. The mixture was centrifuged to equilibrium (Beckman 50.Ti rotor, 38,000rpm, 24 hrs). Phage bands were collected with sterile 22 gauge needles and exhaustively dialysed against phage dialysis buffer (10mM NaCl, 5mM Tris pH 8.0, 10mM $MgCl_2$).

The phage DNA was extracted using two phenol, two phenol/chloroform (1:1), two chloroform and two ether extractions. All organic components were pre-equilibrated with TE. By the addition of 3M sodium acetate (pH 5.0), the phage DNA was precipitated to a final concentration of 0.3M and 2.5vols of 100% ethanol. After chilling for 20 mins at -70°C, or overnight at -20°C, the DNA was pelleted by centrifugation in a microcentrifuge (10,000rpm, 10 mins, 4°C). The pellet was washed twice in cold 70% ethanol and dried very briefly *in vacuo*.

The DNA pellet was finally resuspended in TE buffer and stored at 4°C or at -20°C. The DNA concentration was determined by measuring the absorbance at 260nm and 280nm and its integrity examined by agarose gel electrophoresis.

2.2.17. Radiolabelling of DNA by Random Priming

2.2.17.1. Random Priming

DNA probes from about two hundred to several thousand base pairs in length can be labelled to a very

high specific activity by the technique of random priming (Feinberg & Vogelstein, 1983). For convenience, the solutions used are listed below.

Solutions:

OLB	Solutions A, B and C in a ratio of 2:5:3; store at -20° C; good for three months with repeated freezing and thawing.
Solution A	625µl 2M Tris-HCl pH 8.0; 25µl $MgCl_2$; 350µl SDW; 19µl 2-mercaptoethanol; 5µl 0.1M dCTP; 5µl 0.1M dTTP; 5µl 0.1M dGTP.
Solution B	2M Hepes buffer titrated to Ph 6.6 with NaOH.
Solution C	Hexadeoxyribonucleotides evenly suspended in 3mM Tris-HCl, 0.2mM EDTA pH 7.0 at 90 OD_{260} units/µl stored at -20° C.
BSA	10mg/ml enzyme grade.
dATP	α^{-32}P-dATP, Amersham, 3000Ci/mmol, 10µCi/µl.
Klenow fragment of DNA polymerase	diluted to 1.5 units/µl.

Isolation of DNA for use as probe:

The DNA fragment desired as a probe was cut using the required restriction enzymes and separated in a low melting temperature agarose gel. The desired band was excised from the gel using a scalpel and placed in a pre-weighed microcentrifuge tube. SDW was added at a ratio of 1.5ml SDW to 1g of gel. The capped tube was placed in a boiling waterbath for 7 min and then allowed to equilibrate at 37° C for at least 10 min before labelling. Isolated fragments were stored at -20° C and reboiled for 3 min, equilibrated for 10 min prior to subsequent labelling. If it was unnecessary to isolate the probe from a gel, for instance if a whole plasmid was to be labelled, then the DNA was added directly to the labelling reaction below.

The labelling reaction:

Reagents were added to a microcentrifuge tube in the following order:

SDW	18μl
OLB	5μl
BSA	2μl
DNA	20μl (20ng)
dATP	3μl
Klenow	2μl

The reaction was allowed to run overnight at room temperature. The mixture was boiled for 5 min prior to hybridization.

2.2.17.2. End Labelling of Oligonucleotides

Oligonucleotides were labelled to a high specific activity by means of γ^{32}ATP and T4 polynucleotide kinase. The composition of the labelling mix was: 10pmol of the oligonucleotide, 10pmol of γ^{32}P-ATP (5Ci/mmol), 2μl

of 10 x kinase buffer, and 10 units of T4 polynucleotide kinase in a final volume of 20μl. The 10 x kinase buffer was composed of 500mM Tris-HCl (pH 7.6), 100mM $MgCl_2$ 50mM DTT, 1mM spermidine, 1mM EDTA. The labelling reaction was incubated for 1 hr at 37°C and terminated by the addition of 1μl of 500mM EDTA.

2.2.17.3. Removal of Unincorporated Labelled Nucleotides

In order to remove unincorporated nucleotides, the radiolabelled mixtures were passed through a Sephadex G-50 column (Maniatis *et al.*, 1989). A 1ml column of Sephadex G-50 was equilibrated in STE by centrifugation (Sorvall HB4 rotor, 2,000rpm, 3 mins, 4°C). The composition of STE was: 150mM NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA. The radiolabelling mix was diluted in 100μl STE and applied to the column. Unincorporated [$^{32}\alpha$]-dATP was trapped in the column and the labelled DNA collected after centrifugation (2,000rpm, 2 mins).

2.2.18. Construction of Libraries

Any DNA cloning procedure has four essential parts: 1) a method for generating DNA fragments; 2) a group of reactions which join foreign DNA to the vector; 3) a means of introducing the artificial recombinant into a host cell in which it can replicate; and 4) a method of selecting or screening for a clone of recipient cells that has acquired the recombinant. The strategy used to construct the libraries is summarised in Fig. 2.1.

Parasite DNA and λNM1149 vector DNA (Fig. 2.2) prepared as described before were digested to completion with EcoRI or HindIII restriction enzyme at a ratio of 3:1 (enzyme units: μg DNA) under the conditions specified by the suppliers (BRL).

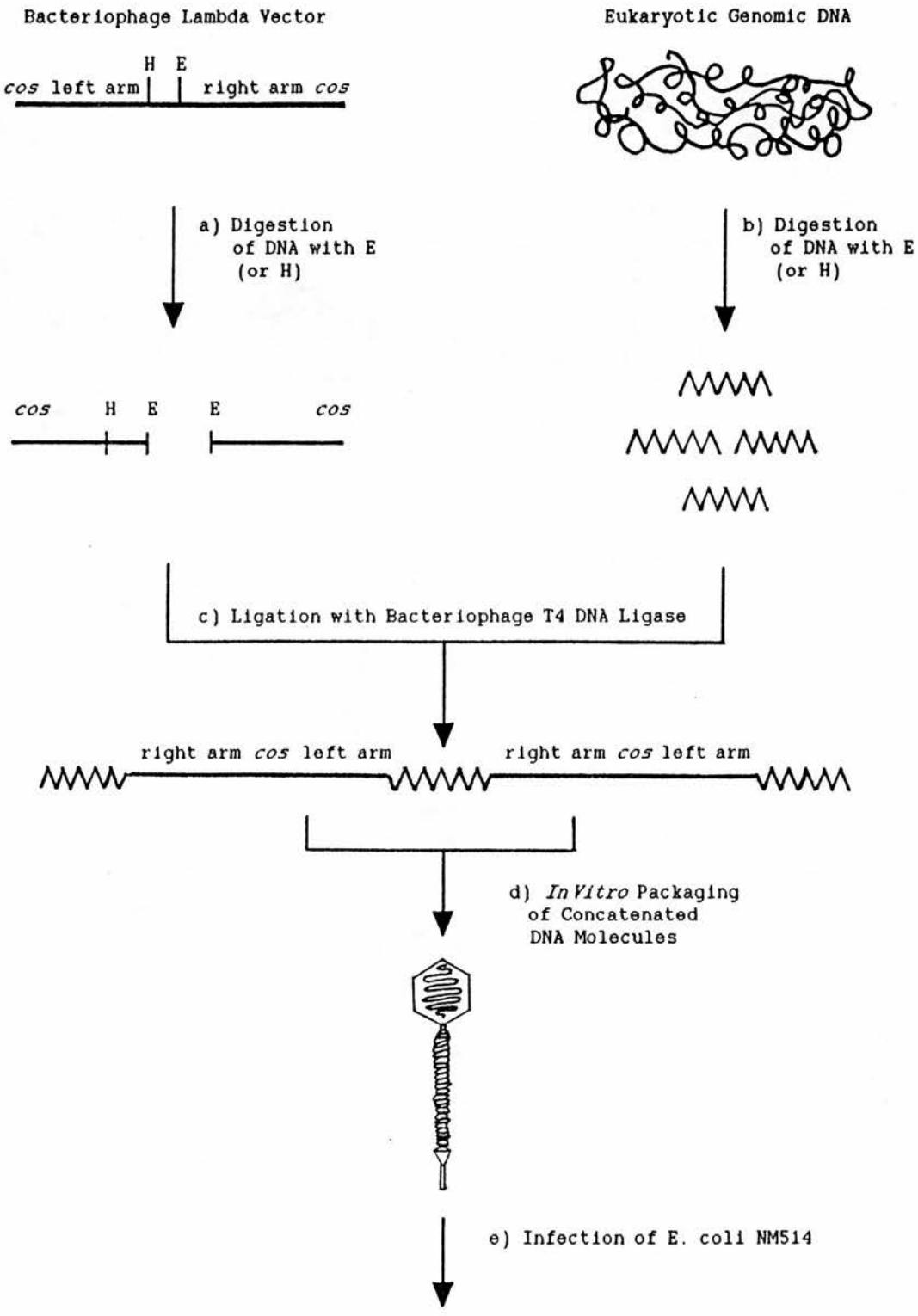


Fig. 2·1 The strategy used to construct libraries of random fragments of eukaryotic DNA. Left: preparation of the vector DNA fragments. Right: preparation of eukaryotic DNA fragments. A concatemeric recombinant DNA molecule is produced by the action of bacteriophage T4 DNA ligase. This concatemer is the substrate for the *in vitro* packaging reaction during which a different recombinant DNA molecule is inserted into each bacteriophage lambda particle. Following amplification by growth in *E. coli*, a lysate is obtained, consisting of a library of recombinant clones that, in aggregate, contain most of the sequences present in the eukaryotic genome. H = HindIII; E = EcoRI. The figure represents digestions with EcoRI.

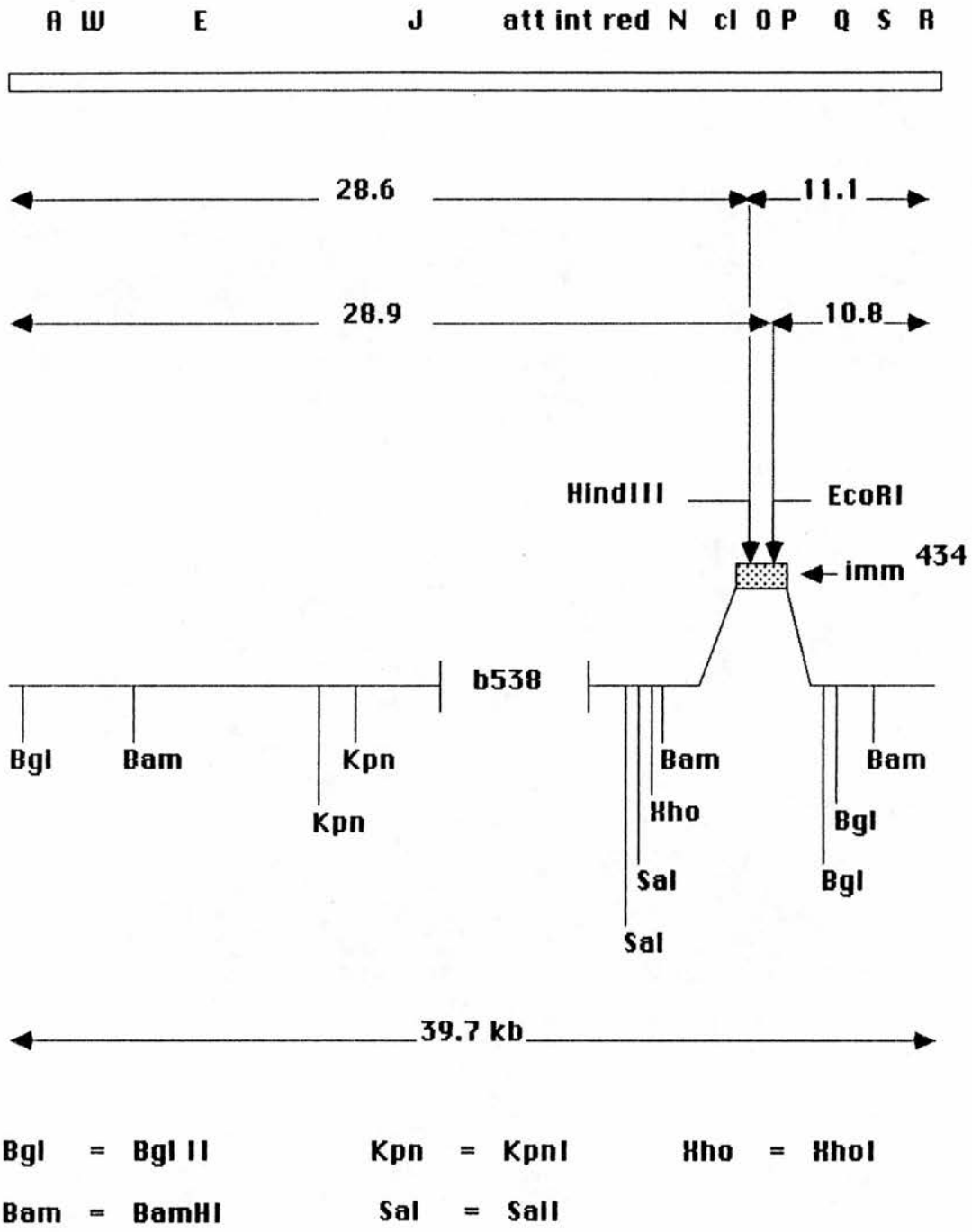


Fig. 2.2 A restriction enzyme map of Lambda NM1149 vector DNA illustrating the unique HindIII and EcoRI cloning sites.

They were then mixed at a 4:1 molar ratio (1μg λ arms: 0.4μg of insert) to a final concentration of 1.4μg of DNA in 10mM ATP, 50 mM Tris-HCl pH 7.5, 10mM $MgCl_2$ and 0.1unit/μl of T4 ligase in a final volume of 10μl. This ligation mix was then incubated at 12°C overnight.

The recombinant DNA was packaged at room temperature for 1h in presence of FTL (freeze-thaw lysate), SE (sonicated extract), and buffers. Both FTL and SE were kindly provided by M. Goman. After 1h at RT, SM buffer was added to stabilise the product phage. The λNM1149 control was plated on Ed8654 and the packaged parasite DNA ligation on NM514 (only recombinants give plaques on this strain).

The number of recombinants in the libraries was 2×10^5 for EcoRI and 2.5×10^5 HindIII.

2.2.19. Library Screening by Hybridisation with DNA or Oligonucleotide Probes

Recombinant phage clones containing sequences homologous to the desired probes were identified by hybridisation to plaques which had been transferred to nylon membranes (Hybond-N).

2.2.20 Phage Lifts

DNA from phage plaques can be transferred to nylon membrane filters by the following procedure. Phage are plated onto dry BBL bottom plates using 0.7% agarose in 10mM $MgSO_4$ instead of BBL top agar and allowed to grow for 6-12 hrs. After cooling at 4°C for 1 hr, a single sheet of nylon membrane, cut to the dimensions of the plate, is laid on the agarose surface for 1 min marking with needle holes for subsequent orientation. The membrane was removed and laid, plaque side up, on blotting paper

soaked in 0.5M NaOH, 1.5M NaCl for 5 mins, then immersed in 0.5M Tris-Cl pH 8.0, 1.5M NaCl for 5 mins, then 2 x SSC for 5 mins and finally air dried. Multiple filters could be prepared from each plate in this manner.

2.2.21. Hybridisation

Different stringency conditions were adopted for the hybridisation with homologous, heterologous or oligonucleotide probes.

2.2.21.1. Hybridisation Condition for Homologous DNA Probe

Filters were pre-hybridised for 1 hr at 60°C in hybridisation solution. The hybridisation solution was composed of 0.5M disodium hydrogen phosphate, adjusted to pH 7.5 with 0.5M sodium dihydrogen phosphate, and 7% SDS. The probe was denatured by boiling for 5 mins and then added to the pre-hybridisation solution. Hybridisation was performed for 18 hrs at 60°C.

After hybridisation the filters were washed at 60°C with shaking in 0.1% SDS, 0.2 x SSC, for 2 hrs with several changes of wash solution. The filters were then air-dried and exposed to X-ray film.

2.2.21.2. Hybridisation Conditions for Heterologous DNA Probe

Filters were pre-hybridised for 1 hr at 37°C in 6 x SSC, 0.4mM EDTA, 0.1% sodium pyrophosphate, 0.2% SDS and heparin (500ug/ml). The probe was added and the hybridisation was performed at 37°C for 18 hrs.

After hybridisation the filters were washed in 6 x SSC at 37°C for 2 hrs with several changes. Further washings were performed in more stringent conditions in

order to obtain less background. Conditions such as 0.4 x SSC, 0.1% SDS were used in our work with heterologous probe.

2.2.21.3. Hybridisation Conditions for Oligonucleotide Probe

The filters were pre-hydridised in the same way as for heterologous probing but the washing was only in 6 x SSC, 0.1% SDS for 2 hrs with several changes. They were air-dried and film exposed.

2.2.22. Identifying and Purifying Recombinants

The desired recombinants were identified on the plates by aligning the signal detected by autoradiography with the plaques on the plate. Positive plaques were picked and resuspended in 1.0ml of phage buffer. Each positive plaque picked was replated so that individual plaques were visible and rescreened by the same procedure. The single positive plaques picked from the third screening were considered to be genetically pure clones.

Phage DNA prep's were performed as in section 2.2.16 for further identification and characterization.

2.2.23 Transfer of DNA to Membrane Filters

2.2.23.1 Southern Blots

This method is essentially as described by Southern (1975), and Maniatis *et al.* (1989). Genomic DNA gels were first treated before transfer. The gel was soaked in 0.25 HCl for 15 mins in order to depurinate the DNA, then in 0.5M NaOH, 1,5M NaCl for 1 hr to denature the DNA and then in 1M Tris-Cl pH 8.0, 1.5M NaCl for 1 hr to neutralize the gel. DNA was transferred to nylon membrane

(Hybond-N, Amersham) by capillary action for 18 hrs using 20 x SSC as transfer buffer in the following blotting sandwich: the gel was placed on a sheet of blotting paper soaked in 20 x SSC and with its ends resting in a reservoir of 10 x SSC; a piece of nylon membrane was cut to exactly the same dimensions as the gel and placed on its surface taking care to avoid trapping air bubbles; a stack of blotting paper about 3cm high was again cut to exactly the same dimensions as the gel; some sheets were pre-soaked with 2 x SSC and placed on top of the membrane followed by the rest of the stack. The transfer was left overnight. After this, the blot was dismantled. The membranes were rinsed in 2 x SSC and air dried. The nylon membrane filters were exposed to ultra-violet light from a gel transilluminator for 3 mins.

2.2.23.2 Dry Blots

A simplified version of the above method (Smith & Summers, 1980) has also been employed. The gel was also denatured in 0.5M NaOH, 1.5M NaCl, but in this case neutralised in 1M ammonium acetate, 0,02M NaOH. The transfer is assembled as described above, except that it requires no wicks of buffer reservoir, and can be bidirectional.

2.2.24. Polymerase Chain Reactions (PCR)

The polymerase chain reaction (PCR) is a simple and powerful method which can selectively amplify a targeted segment of DNA *in vitro* (Saiki *et al.*, 1985). PCR is based on the enzymatic amplification of a DNA fragment that is flanked by two oligonucleotide primers which hybridise to opposite strands of the target sequence. The primers are orientated with their 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary

sequences and extension of the annealed primers with Taq DNA polymerase results in the amplification of a DNA fragment defined by the 5' ends of the primers. This process can amplify a single molecule of template DNA a million fold.

A modification of the PCR procedure of Saiki *et al.* (1985) and Mullis & Faloona (1987) was adopted. The reaction mixture was typically composed of either 200ng of *O. gibsoni* genomic DNA or 1ng of the recombinant vector DNA, 10µl of a 1µM solution of each primer, 2µl of a 10mM solution of each dNTP, 10µl of a 10 x reaction buffer (500mM KCl, 100mM Tris-HCl pH 8.3, 15mM $MgCl_2$, 0.01% gelatin), to give a final volume of 99.5µl. Finally, to this was added 0.5µl of Taq DNA polymerase (5u/µl; purchased from Perkin Elmer Cetus). To reduce evaporation or refluxing the reaction mix was overlayed with 100µl of mineral oil (Sigma). DNA segments were amplified after 30 cyclical temperatures of 92°C (denaturation), 40° (annealing), and 70°C (extension) in a heating block (purchased from Hybaid).

2.2.25 Cloning into vectors

The well-isolated clones were subcloned into the plasmid pUBS or into M13 for sequencing and further characterization.

2.2.25.1. Cloning into pUBS

Following digestion of 2µg of pUBS vector (Fig. 2.3) DNA with the appropriated enzyme(s), the restriction was checked on a mini gel. The digest was then phenol extracted, ethanol precipitated, dried *in vacuo* and redissolved in 1 x TE to a final concentration of 100ng/µl. The fragment to be cloned was then ligated to the vector at an insert:vector molar ratio of 3:1 in a

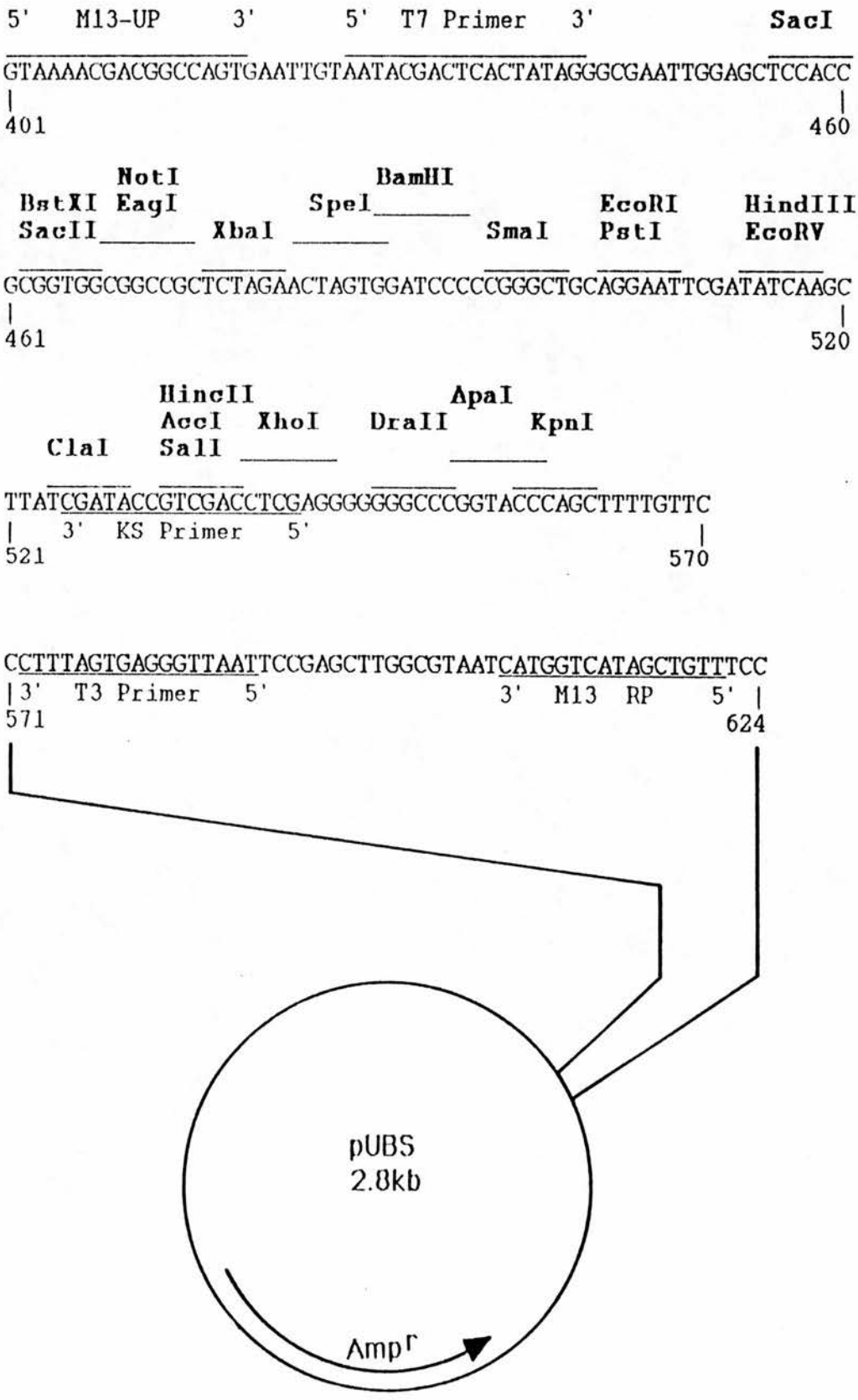


Fig. 2.3. pUBS Plasmid Multiple Cloning Sites.

final volume of 10µl. This volume was composed of 1µl of 10 x ligation buffer (500mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 100mM DTT, 100mM ATP), and 0.5units of T4 DNA ligase. The ligation mixture was incubated at 15°C overnight.

The ligated pUBS vector was introduced into the JM83 host via transformation of competent cell as described in section 2.2.11.

The transformed cells were plated out in L-Broth supplemented with 50µg/ml of ampicillin and the plate incubated overnight at 37°C.

The insertion of a DNA fragment into the multiple cloning site of the pUBS does not allow an easy identification of cells harbouring recombinant plasmid. For all the cells carrying the plasmid (recombinant or non-recombinant) will grow in L-Broth-amp plates. Thus, the only way to identify the recombinants is by the hybridisation procedure using the desired insert as probe.

2.2.25.2. Cloning into M13

The appropriate DNA fragments are cloned into M13 vectors essentially as described for pUBS vector (section 2.2.24.1), as well as for the transfection in TG1 cells. The plating procedure was on minimal media by adding 2,5ml of top agar containing 30µl Of X-gal (20mg/ml in dimethylformamide), 20µl of IPTG (24mg/ml in H2O) and 0.2ml of stationary phase cells. Plates were incubated at 37°C.

E. coli host cells infected with any M13 vector strains produce, in the presence of the *lac* operon inducer IPTG (isopropyl-ß-D-thiogalactopyranoside), a functional ß-galactosidase. Such cells hydrolysed the substrate X-gal (5-bromo-4-chloro-3-indolyl-ß-galactoside) to give a blue

dye (bromochloroindole). Insertion of foreign DNA into an appropriate site in the DNA vector interfered with the production of ß-galactosidase in infected cells. Thus recombinant M13 gave colourless plaques on an *E. coli* lawn in contrast to the blue plaques given by the intact vector. The colourless recombinant plaques were picked out and grown quickly to give single-stranded template for Sanger 'dideoxy sequencing'.

2.2.26. Dideoxynucleotide DNA Sequencing

In order to sequence in conformity with the dideoxynucleotide chain termination principle of Sanger *et al.* (1977), it was necessary to isolate the DNA to be sequenced in a single stranded form. This was made by cloning the DNA fragments into the M13mp series of phage vectors from which the single stranded DNA could be prepared (Messing *et al.*, 1981). Chen & Seeburg (1985) have developed a technique for sequencing supercoiled plasmid DNA which makes it possible to use double stranded DNA for sequencing in compliance with Sanger's method. For convenience, the sequencing solutions are listed below.

2.2.26.1. Solutions

Sequenase Buffer (5 x concentrate)
200mM Tris-HCl pH 7.5
100mM $MgCl_2$
250mM NaCl

Control DNA M13mp18 (0.2 µg/µl)

Dithiothreitol (0.1M)

Labelling Mix (dGTP) 5 x concentrate (GREEN tube)
7.5µM dGTP
7.5µM dCTP

7.5μM dTTP

Labelling Mix (dITP) 5 x concentrate (YELLOW tube)
15μM dGTP
7.5μM dCTP
7.5μM dTTP

ddG Termination Mix (for dGTP, RED tube)
80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP
8μM ddGTP, 50mM NaCl

ddA Termination Mix (for dGTP, RED tube)
80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP
8μM ddATP, 50mM NaCl

ddT Termination Mix (for dGTP, RED tube)
80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP
8μM ddTTP, 50mM NaCl

ddC Termination Mix (for dGTP, RED tube)
80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP
8μM ddCTP, 50mM NaCl

Sequence Extending Mix (for dGTP, CLEAR tube)

ddG Termination Mix (for dITP, ORANGE tube)
160μM dITP, 80μM dATP, 80μ, dCTP, 80μM dTTP
1.6μM ddGTP, 50mM NaCl

ddA Termination Mix (for dITP, ORANGE tube)
80μM dITP, 80μM dATP, 80μM dCTP, 80μM dTTP
8μM ddATP, 50mM NaCl

ddT Termination Mix (for dITP, ORANGE tube)
80μM dITP, 80μM dATP, 80μM dCTP, 80μM dTTP
8μM ddTTP, 50 mM NaCl

ddC Termination Mix (for dITP, ORANGE tube)

80μM dITP, 80μM dATP, 80μM dCTP, 80μM dTTP
8μM ddCTP, 50mM NaCl

Sequence Extending Mix (for dITP, CLEAR tube)

Sequenase Version 2.0 Enzyme (BLUE tube)

Enzyme Dilution Buffer

10mM Tris-HCl pH 7.5
5mM DTT
0.5mg/ml BSA

Stop Solution

95% Formamide
20mM EDTA
0.05% Bromophenol Blue
0.05% Xylene Cyanol FF

10 x TBE

0.9M Tris-borate pH 8.3, 25mM EDTA

40% Acrylamide

380g Acrylamide
20 g bis-acrylamide made to 1l with dH_2)
Deionised with 20g MB-1 (BDH) and filtered.

Gel Fix

10% Glacial acetic acid
10% Methanol in dH_2O

2.2.26.2. Sequencing Gel Electrophoresis

Previously siliconised glass plates, measuring 20 x 50 cm, were meticulously cleaned with a soapy sponge, rinsed thoroughly with distilled water, rinsed with ethanol and carefully wiped dry to remove any residual particles. The gel sandwich was built using the two glass plates separated by 0.35mm thick x 1.0cm wide teflon spacers. The plates were taped together, side and bottom, with 3.1cm wide yellow electrical tape. In order to prevent leakage, the sandwich bottom was taped once again, forming "hospital bed corners".

A standard 8% polyacrylamide/urea sequencing gel was prepared in the following way: 10ml 40% acrylamide stock, 25g urea, 5ml 10 x TBE, and 15ml dH_2O. The solution was stirred to dissolve the urea. It was then filtered and de-gassed under vacuum. Immediately before pouring 15μl of TEMED (N,N,N^1,N^1-tetramethyl-ethylene diamine) was added together with 350μl of 10% ammonium persulphate.

The gel was poured by means of a 25ml pipette to deliver the solution between the glass plates. The plates were kept at a low angle during pouring and tilted slightly so that the gel solution could move slowly down one side. Air bubbles were prevented by pouring the gel solution at a steady pace while maintaining constant contact between the fluid being poured and the fluid already between the plates. Any air bubbles that did form were removed by tapping the glass behind the bubbles. The flat edge of a sharkstooth comb was then inserted between the plates to a depth of 2-3mm below the short plate.

When the gel was set, the tape from the bottom of the sandwich was removed. The top of the gel was flooded with distilled water and any extraneous polyacrylamide removed from around the comb with a razor

blade. The comb was then placed between the glass plates with the teeth down towards the gel to form the sample wells. The gel sandwich was clamped into the electrophoresis chamber and the upper and lower reservoirs filled with 1 x TBE electrophoresis buffer. Gels were pre-electrophoresed for 15 mins before loading the samples. Prior to loading the sample, it was important to rinse the wells to remove the urea that had diffused into the well (if this is not done, the sample does not form a tight band; thus, band sharpness on the auto-radiograph will suffer). Gel electrophoresis was performed at a constant power, usually 40 watts, for an adequate length of time.

After electrophoresis, the gel was dismantled in a way such that it remained attached to one plate (non-siliconised) and then transferred to a tray of gel fix for 15 mins. Gels were transferred to Whatman 3mm blotting paper, covered with Saran Wrap and vacuum-dried at 80°C on a gel drier. Dried gels were placed directly in contact with X-ray film overnight or for several days before processing.

2.2.26.3. Sequencing of Single Stranded Recombinant M13mp DNA

2.2.26.3.1. Preparation of Single Stranded DNA Templates

A fresh M13 recombinant white plaque was added to 1.5ml of a 1/50 dilution of an overnight culture of TG_1 cells. The cells and phage were incubated at 37°C for 5-6 hrs with constant shaking. The cells were harvested in a microcentrifuge and the supernatant containing the single stranded phage was decanted into a fresh microcentrifuge tube. In order to remove any residual cells, the supernatant was centrifuged once again. To precipitate the phage 300μl of 20% PEG 6000, 2.5M NaCl were added for 1.2ml of supernatant and incubated at room temperature for

30 mins. The tube was microcentrifuged for 15 mins to collect the phage precipitate. The supernatant was discarded and the tube microcentrifuged for a few moments to collect the residual supernatant which was then taken off with a drawn pasteur on a vacuum line. The phage pellet was resuspended in 100μl of TE supplemented with 0.1M NaCl. The phage were extracted with an equal volume of phenol by vortexing the microcentrifuge tube for 10 s and then standing for 2 mins. The cycle was repeated twice more and the tube microcentrifuged for 5 mins. The aqueous layer was carefully removed and re-extracted with an equal volume of phenol-chloroform (1:1) mix. Again the sample was microfuged and the aqueous phase removed. The template DNA was precipitated by the addition of one tenth the volume of 3M sodium acetate pH 4.8 and two and a half volumes of 100% ethanol. After incubating at 70°C for 30 mins the DNA precipitated was pelleted by spinning in a microcentrifuge for 15 mins. The pellet was washed with 1ml of 70% ethanol, vacuum dried, and resuspended in 40μl of TE.

2.2.26.3.2. Sequencing Reactions

Initially, the sequencing primer was hybridised to the template DNA. To a microcentrifuge tube were added 5μl of the single stranded template DNA (0.5-1.0μg), 2μl of the sequencing primer (4ng), 1μl TM buffer, and the volume adjusted to 10μl with double distilled water. The annealing mix was treated at 65°C for 5 mins to denature and the tube was then allowed to equilibrate to room temperature for 30 mins. The slow cooling step allowed the primer to anneal to the template.

During the primer annealing reaction 2μl of each of the four ddNTP termination mixes were added to separate microcentrifuge tubes labelled G, A, T, and C.

After equilibration of the temperature of the annealing mix, 1μl 100mM DTT, 1μl Large Fragment DNA Polymerase I (Klenow Fragment, 1 unit/μl) were added. Following gentle mixing 3μl aliquots were dispensed into the four tubes labelled G, A, T, and C containing the termination mixes. After collecting by brief centrifugation to form a single drop the termination reactions were incubated at 30°C for 20 mins. Immediately proceeding this 2μl of sequencing chase was added to each tube and again the chase reaction incubated at 30°C for 20 mins. The reactions were halted by adding 2μl of stop buffer. Before the reactions were loaded onto a sequencing gel they were first denatured by heating at 65°C for 5 mins and quickly transferred to ice for 1 min.

2.2.26.4. Sequencing of Double Stranded Recombinant Plasmid and Lambda DNA

The instability of some M13 clones containing intronic sequences determined a change in the strategy of sequencing so that double-stranded sequencing in plasmid pUBS (Fig. 2.3) and even λNM1149 could be employed successfully.

The greatest difficulties with sequencing double-stranded DNA are: 1) the DNA must be purified thoroughly or background and ambiguity problems will be encountered; 2) the denaturing and neutralization steps also can generate the same inconvenience if some double-stranded DNA remains in the reaction. The methods previously described for plasmid and lambda DNA preparations were employed, as well as the spin-dialysis sepharose (Sigma CL-6B-200) column which also gave excellent results.

2.2.26.4.1. Solutions, Reagents, Mixes, and Primers.

For the spin-dialysis, the sepharose-6CBL (Sigma) was equilibrated in $TE_{0.1}$ to a ratio of 2 parts gel to 1 of buffer per volume. A 0.5ml eppendorf tube was pierced with a 21G syringe needle but just the tip of the needle emerged though the bottom. 20μl of water-washed sterile 200 micron glass beads (Sigma G-1145) were added to the 0.5ml tube, followed by 0.2ml of sepharose. This tube was placed inside a 1.5ml eppendorf tube also pierced and the set put inside a suitable centrifuge tube for a low centrifugation (Sorvall HB4 rotor, 1,000rpm, 4 mins). After this, the 1.5ml tube was replaced by an intact one and the DNA sample added to the top of the sepharose and then re-spun as previously described.

This process was used whenever DNA had to be separated from salts or other small molecules. It is an alternative to ethanol precipitation after phenol/ chloroform extraction of DNA. The column size was adjusted to the volume of the sample, that is, the packed column volume had to be four times the sample volume.

Solutions:

TM Buffer (10+)	70mM Tris-HCL-7.5
	70mM MgCl2
	300mM NaCl
	100mM DTT
	1mM EDTA
$TE_{0.1}$	10mM Tris-HCL-7.5
	0.1mM EDTA

ddNTP Termination mixes

	A	C	G	T
0.5mM dCTP	500µl	25µl	500µl	500µl
0.5mM dGTP	500µl	500µl	25µl	500µl
10mM ddATP	1µl			
10mM ddCTP		8µl		
10mM ddGTP			16µl	
10mM ddTTP				50µl
$TE_{0.1}$ buffer	500µl	1000µl	1000µl	1000µl

(Obs.: 25µl aliquots were dispensed into tubes; the amount was sufficient for 10 clones; unused mixture was restocked)

Chase-mix — 0.5mM dGTP; 0.5mM dATP; 0.5mM dTTP; 0.5mM dCTP in H_2O.

Denaturing Solution — 1N NaOH; 1mM EDTA.

Klenow/^{35}S-ATP mix — 1µl of 10 x TM; 7.5µl of H_2O; 0.5µl large fragment DNA Polymerase (Klenow fragment, 5units/µl); and 1µl of 35S-dATP.

Sequencing primers:

Oligonucleotide sequencing primers were synthesised by Oswell DNA Service, Department of Chemistry, University of Edinburgh.

M13 Universal sequencing primer

5'-GTA AAA CGA CGG CCA GT-3'

M13 Reverse sequencing primer

5'-AAC AGC TAT GAC CAT G-3'

ß-Tubulin internal oligonucleotide #0

5'-ATA GTA AAG CGT GAA TGA AT-3'

ß-Tubulin internal oligonucleotide #1

5'-TAC CGC GGA TCG ATC CA-3'

ß-Tubulin internal oligonucleotide #4

5'-TTA TAT TCT CGG GCC AAC TA-3'

ß-Tubulin internal oligonucleotide #5

5'-CAA ATT AAT ATA AAT ACC TC-3'

ß-Tubulin internal oligonucleotide #6

5'-TTC AAT TCA AAC TTA TG-3'

ß-Tubulin internal oligonucleotide #7

5'-GCG CAT ATA GTG CAG TT-3'

ß-Tubulin internal oligonucleotide #8

5'-GGC TGC AAC ATT TAT TGG-3'

ß-Tubulin internal oligonucleotide #9

5'-GGT GGA TCA ATA ATT TA-3'

ß-Tubulin internal oligonucleotide #10

5'-ACA TCA TTC GTA CTA TA-3'

ß-Tubulin internal oligonucleotide #11

5'-AGA TGA AAC TAA TAA TTG C-3'

ß-Tubulin internal oligonucleotide #A

5'-AAT TCA TCT ACA ATC CC-3'

ß-Tubulin internal oligonucleotide #A2

5'-TGA GGT AAT TGA TAT GC-3'

ß-Tubulin internal oligonucleotide #B

5'-GTT GAA ACC TTA GAA CC-3'

2.2.26.4.2. Sequencing reactions

10µg of DNA (plasmid or lambda) in a volume of 20µl were denatured with 5µl of denaturing solution, for 10 mins at room temperature. The sample was spin-dialysed though sepharose-6BCL equilibrated in $TE_{0.1}$ and the annealing step performed in presence of primer and TM buffer in the following proportion: 8µl of template DNA (5-10µg); 1µl of sequencing primer (4ng) and 1µl 10 x TM buffer.

The mixture was incubated for 15 mins at 37°C, then immediately used for the sequencing reactions. 2.5µl

of the primed template was dispensed into each of the four reaction tubes containing 2μl of the appropriate ddNTP mix and 2μl of Klenow/35S-dATP was added. The initial reaction was performed for 10 mins at 42°C, then 2μl of chase-mix (0.5mM of each dNTP) were added. The reaction was carried on for 5 mins at 42°C, terminated with 4μl of formamide dye mix, and denatured by boiling for 2 mins before loading on gel.

2.2.27. DNA Sequence Data Compilation and Analysis

Sequence information was obtained from the autography and compiled by version 2.0 of the programs of Staden and analyzed by version 4.0 of the programs of the University of Wisconsin Genetic Computer Group (Devereux *et al.*, 1984).

CHAPTER 3
RESULTS AND DISCUSSION

3.1. IDENTIFICATION OF ß-TUBULIN GENE IN *O. gibsoni*

As discussed in Chapter 1, the analysis of all the available ß-tubulin sequences unambiguously reveals the existence of a highly conserved polypeptide framework in which individual sequences typically diverge from each other both within and between species in 2-8% for the isotypic classes, and in 8-25% for non-isotypic forms of ß-tubulins (from approximately 430 residue positions excluding the more divergent marginal C-terminal region). However, the carboxyl-terminal (approx. 15 residues) constitutes a major variable region domain for ß-tubulin. In this short region, substitutions, deletions, and terminal additions of amino acids are found in >75% of the residue positions.

This stringent intraspecies conservation of ß-tubulin genes allowed us to use a ß-tubulin gene fragment from *Plasmodium falciparum* (Fig. 3.1) as a probe in the identification of the ß-tubulin gene from the filarial worm *O. gibsoni*. In addition, the high content of A-T in the base composition of *Plasmodium* DNA surely has helped in the heterologous hybridisation since that remarkable feature was also present in *O. gibsoni* genome.

As a first step towards the cloning and characterisation of the *O. gibsoni* ß-tubulin gene, it was necessary to establish the hybridisation conditions for the heterologous probe in order to find the number and size of the genomic restriction fragments which the gene may span.

Thus, 10µg of genomic DNA from *O. gibsoni* were digested with the restriction enzymes HindIII and/or EcoRI, fractionated by agarose gel electrophoresis and then transferred to Hybond-N by the technique of Southern Blotting for further hybridisation. A 0.5kb *P. falciparum*

ß-tubulin fragment (KpnI-HindIII fragment from exonII -- Fig. 3.1), kindly supplied by Delves *et al.* (1989), was used as a probe.

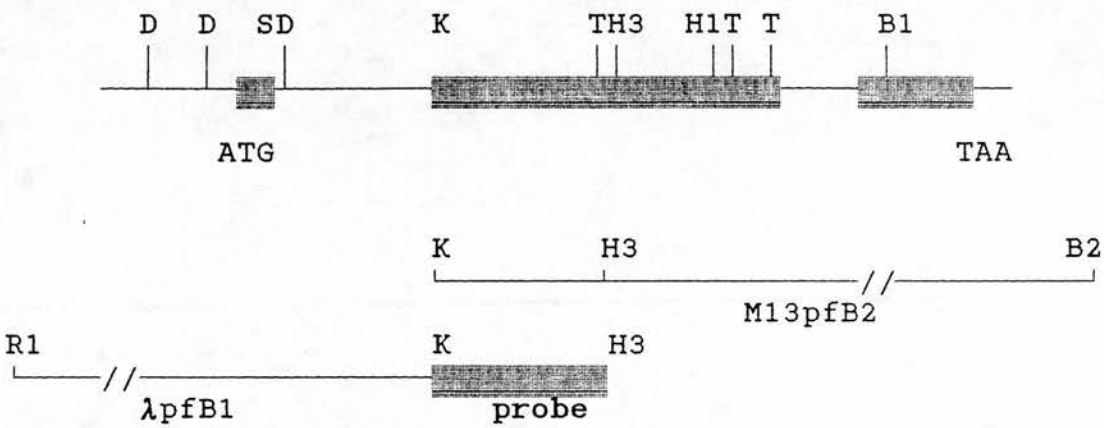


Fig. 3.1. Restriction map of the ß-tubulin gene of *P. falciparum*. The fragment Kpn/HindIII, from exon II, was utilised as a probe to isolate the recombinants λNM1149 for the ß-tubulin gene from a *O. gibsoni* genomic library. ATG indicates the start codon and TAA the stop codon for the ß-tubulin gene in *P. falciparum*. The boxed regions represent the DNA coding region for the protein. Enzyme abbreviations are: B1, BclI; B2, BglII; D, DraI; R1, EcoRI; H1, HpaI; H3, HindIII; K, KpnI; S, Sau3A; T, TaqI. (Delves *et al.*, 1989).

The best hybridisation condition was found to be in 4 x SSC, 500ug/ml heparin, 0.1% $Na_2P_2O_7$, 0.2% SDS at 37°C with a washing stringency of 0.4 x SSC, 0.1% SDS at 37°C (section 2.2.21.1).

The *Plasmodium* ß-tubulin fragment hybridised to at least 4 bands in the HindIII, EcoRI, and EcoRI/HindIII restriction enzyme digests (Fig. 3.2.A and B). The hybridisation patterns obtained in the EcoRI genomic digests revealed a very strong band at about 3.6kb in size.

FIGURE 3.2.A.

Ethidium bromide stained agarose gel of digested *O. gibsoni* genomic DNA prior to Southern blotting.

Track A: HindIII
Track B: EcoRI
Track C: HindIII/EcoRI
Track M: λ HindIII DNA markers
(23.1, 9.4, 6.7, 4.4, 2.3, and 2.0kb)

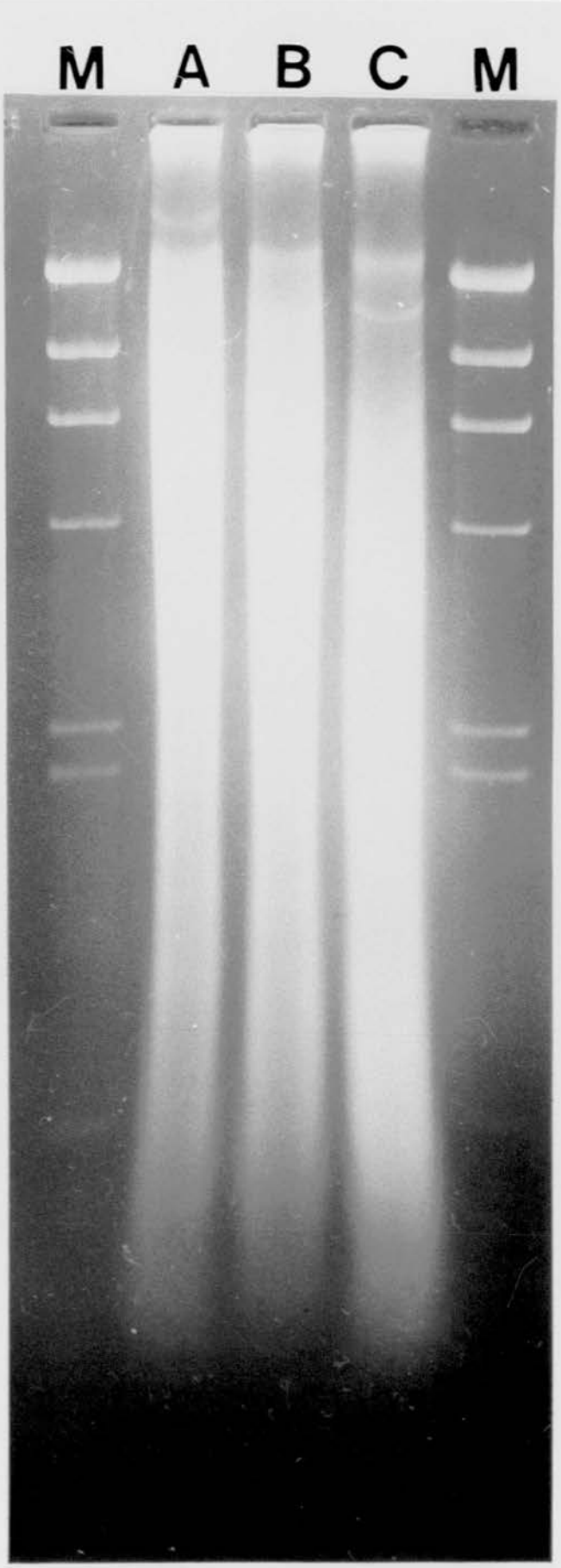
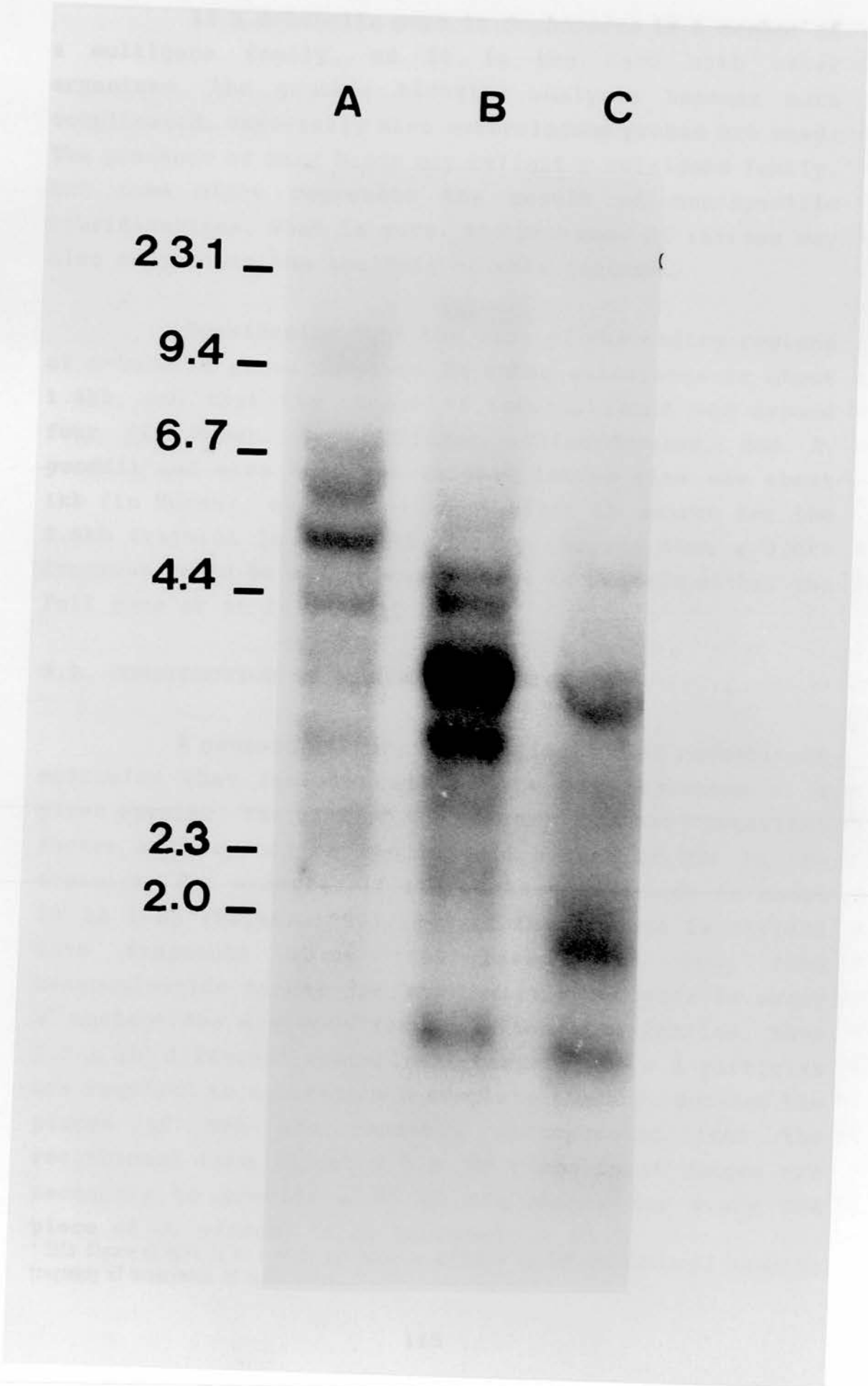

M
A
B
C
M

FIGURE 3.2.B

Southern blot of HindIII (track A), EcoRI (track B), and HindIII/EcoRI (track C) genomic digests probed with a ß-tubulin gene fragment from P. *falciparum*.

Hybridisation was carried out at 37°C in 4 x SSC, 500µg/ml heparin, 0.1% $NaPyrPO_4$, 0.2% SDS with a washing stringency of 0.4 x SSC, 0.1% SDS at 37°C. Overnight exposure.


A
B
C
23.1
9.4
6.7
4.4
2.3
2.0

If a ß-tubulin gene in *Onchocerca* is a member of a multigene family, as it is the case with other organisms, the genomic blotting analysis becomes more complicated, especially when heterologous probes are used. The presence of many bands may reflect a multigene family, but some might represent the result of non-specific hybridisations. What is more, the presence of introns may also complicate the analysis of this pattern.

Considering that the size of the coding regions of ß-tubulin genes observed in other eukaryotes is about 1.4kb, and that the number of introns found was around four (in Human, Rat, Chicken, *Chlamydomonas*, and *T. gondii*) and also that the largest intron size was about 1kb (in Human), we found it convenient to search for the 3.6kb fragment in an EcoRI library, hoping that a 3.6kb fragment would be sufficiently large to contain either the full gene or at least most of it.

3.2. CONSTRUCTION OF GENOMIC LIBRARIES

A genomic library is a collection of recombinant molecules that includes all of the DNA sequences of a given species. The size of the library is a very important factor and depends on the haploid amount of DNA in the organism. For example, if the *Onchocerca* genome is about 10^5 kb long (Rajan, 1990), and if this genome is divided into fragments about 4096 base pairs long (the hexanucleotide target for EcoRI will occur once in every 4^6 nucleotides = 4096*) for insertion into λNM1149, then 2.5×10^4 different recombinant bacteriophage λ particles are required to constitute a complete library. Because the pieces of DNA are randomly incorporated into the recombinant form, about 2.5×10^5 recombinant phages are necessary to provide a 90 to 95% chance for every DNA piece of *O. gibsoni* to be included.

* This figure of 4096 is an average but makes no allowance for A-T bias which would increase the frequency of occurrence of EcoRI sites.

Lambda NM1149 (Murray, 1983) is a phage designed for cloning EcoRI or HindIII fragments (Fig. 2.2; section 2.2.18). It has the kind of deletion termed B538, allowing up to 11kb of foreign DNA to be inserted. The EcoRI and HindIII cloning sites are both located in the cI gene of the phage. Thus when DNA is inserted into either of these sites the recombinants have an inactive cI gene. As a result, they cannot make a repressor and are obliged to enter the lytic cycle. These can be selected on an appropriate bacterial strain (NM514). The phage λ NM1149 is therefore very useful for selecting genuine recombinants when the insert DNA is in short supply. The strategy for the construction of a library in λ NM1149 is shown in fig 2.1 (section 2.2.18).

Since it appeared that the 3.6kb fragment in the genomic blot, EcoRI digested, contained a ß-tubulin related sequence, it was decided to build an EcoRI genomic library in the vector λ NM1149 and use the heterologous probe from *P. falciparum* to screen for phages containing the desired gene.

Two libraries, EcoRI and HindIII, were constructed in λ NM1149 as described in chapter 2 and as outlined in Fig. 2.1. This method had a number of advantages over other approaches: 1) λ NM1149 as a vehicle for the replication of a foreign DNA molecule is a particularly suitable vector, since it can be easily grown, purified and manipulated *in vitro*; 2) it allows up to 11kb of foreign DNA to be inserted, and this is a reasonable size for further manipulations; 3) packaging of λ DNA is generally more efficient than transformation of *E. coli* with plasmids, because the former yields a bigger number of recombinants for an equal amount of DNA; 4) a positive selection of recombinants is obtained by plating the phages on *E. coli* NM514; and 5) λ plaques can be screened at a much higher density than *E. coli* colonies.

Using the procedures described in section 2.2.18, we generated two genomic libraries that contained 2 x 10^5 recombinants for the EcoRI library and 2.5 x 10^5 recombinants for the HindIII one. Before amplification, the EcoRI genomic library was used for the isolation of repetitive DNA sequences since such procedure would to be able to supply information about the quality of the library.

3.3. SCREENING OF GENOMIC *O. gibsoni* LIBRARY FOR REPETITIVE DNA CLONES

DNAs from virtually all eukaryotes contain families of repetitive sequences (Britten & Kohne, 1968). These sequences, which range in length from 130 to 300 base pairs (bp), depending on the species, may be present in many thousands of copies per genome.

Since repetitive DNA sequences do not encode functional products, they are not constrained to retain their primary sequence and diverge rapidly, so that the sequence of a repeat from an organism is often significantly different from the homologous element even in a closely related organism. In addition, their abundance in the genome means that every unit amount of genomic DNA from an organism has a greater mass of a repeat DNA element than any unique copy sequence. To take just one example, it was mentioned earlier that 20% of the *B. malayi* genome is composed of repetitive DNA; a single family of repetitive DNA in this organism, HhaI family, comprises 8% of its genome. *Onchocerca* appears to contain at least one family of tandem repetitive DNA, with a 149 bp monomer repeated throughout the genome (Meredith *et al.*, 1989).

Considering the abundance of such elements, it is possible to check the quality of the library by using

^{32}P labelled total genomic DNA from *O. gibsoni* as a probe. The number of clones carrying such repetitive sequences can supply information on how representative is the library.

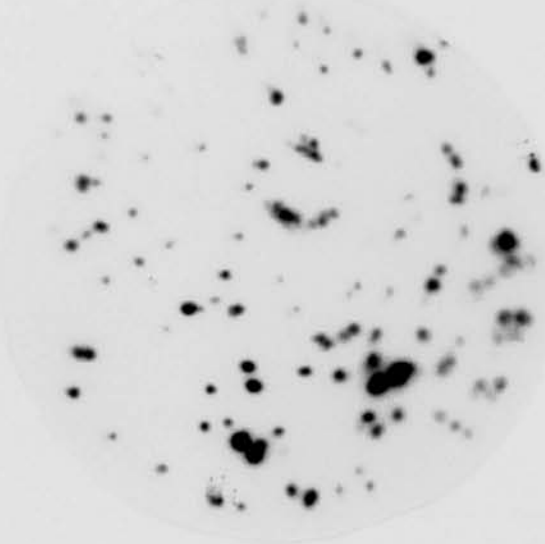
Probing the EcoRI library with labelled total *O. gibsoni* genomic DNA, positive plaques were detected at a rate of around 100 in 1000 phages, with wide ranging of signal strength. 12 of these plaques, 6 very strong and 6 weak ones, were selected for a second screening. The result of the first and second screening shows that the library was representative enough to be used for our purpose (Fig. 3.3.A, B).

FIGURE 3.3.

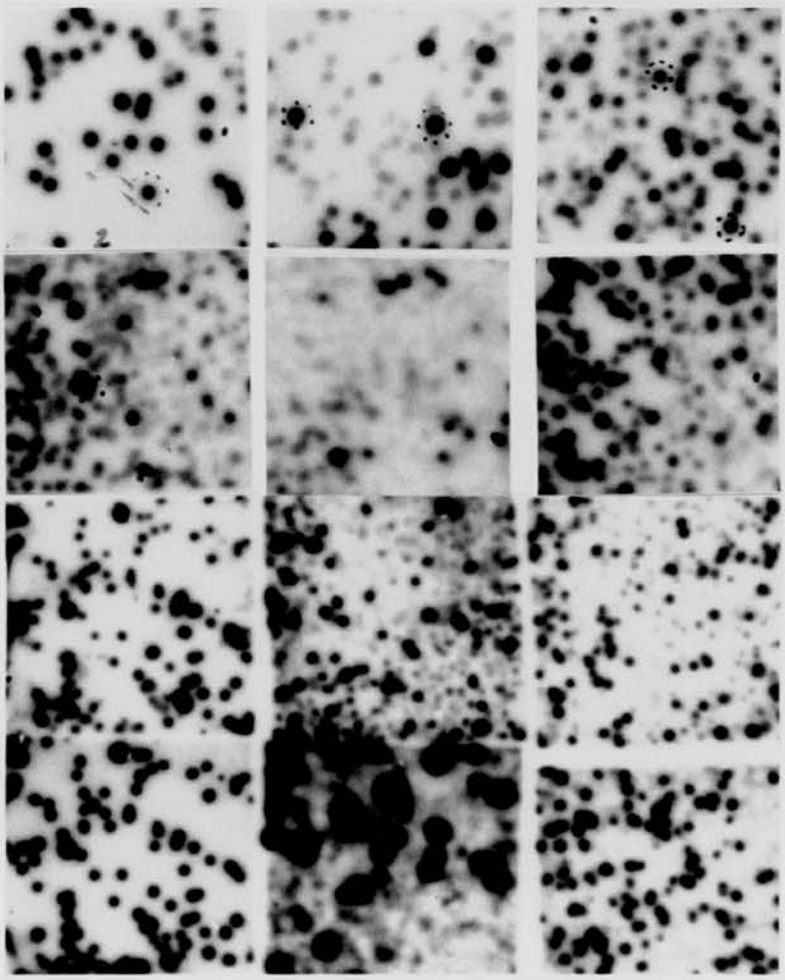
Isolation of Repetitive DNA clones. Autoradiography of plaque lifts from the *O. gibsoni* genomic EcoRI library probed with labelled total *O. gibsoni* genome DNA.
(A) First screening (circular filter).
(B) Second screening (square filters).

Hybridisation was carried out at 37°C, in 4 x SSC, 0.4mM EDTA, 0.1% $NaPyrPO_4$, 0.2% SDS and heparin (500μg/ml). The washing was at 37°C in 0.4 x SSC and 0.1% SDS. The film was exposed overnight.

A



B



3.4. ISOLATION OF THE GENOMIC ß-TUBULIN CLONE FROM EcoRI LIBRARY

The *Plasmodium* ß-tubulin probe recognised 4 large fragments in HindIII genomic digests and hybridised very strongly to a 3.6kb fragment in EcoRI genomic digests of *O. gibsoni* DNA. The EcoRI fragment, unlike the HindIII ones, had an appropriate size to be easily accommodated into the cloning vector λ NM1149 in a greater frequency. Therefore, the EcoRI library was the first to be used for screening with *Plasmodium* probe.

During the isolation of the genomic ß-tubulin clones from the EcoRI library, positive plaques were detected at the rate of approximately one in ten thousand recombinant phages (Fig. 3.4). As the size of the filarial worm genome is about 10^5kb, a single copy gene would be present at a level of approximately one in 2.5 x 10^4 recombinant phage (assuming that most of the recombinants contain a 4kb insert). The 16 plaques giving positive signals in the first screening were picked, enriched, and purified for further characterization (Fig. 3.5).

Clearly the copy number of ß-tubulin gene in *O. gibsoni* needs to be further investigated in a more direct way. The general impression is that either the pattern of bands in the genomic blot or the 9 (out of 16) positive clones resulting from the tertiary screening suggests that we are dealing with a multigene family.

3.5. ANALYSIS OF THE ß-TUBULIN GENOMIC CLONES

DNA was prepared from the 16 phage lambda clones, digested with EcoRI to liberate the insert, fractionated by agarose gel electrophoresis, transferred to Hybond-N membrane, and re-probed with *the Plasmodium*

FIGURE 3.4

Isolation of the genomic ß-tubulin EcoRI clone. Autoradiography of plaque lifts from the *O. gibsoni* genomic EcoRI library probed with *P. falciparum* ß-tubulin 0.5kb fragment (from exon II).

Hybridisation was carried out at 37°C, in 4 x SSC, 0.4mM EDTA, 0.1% NaPyrPO4, 0.2% SDS and heparin (500µg/ml). The washing was at 37°C in 0.4 x SSC and 0.1% SDS and film exposed overnight.

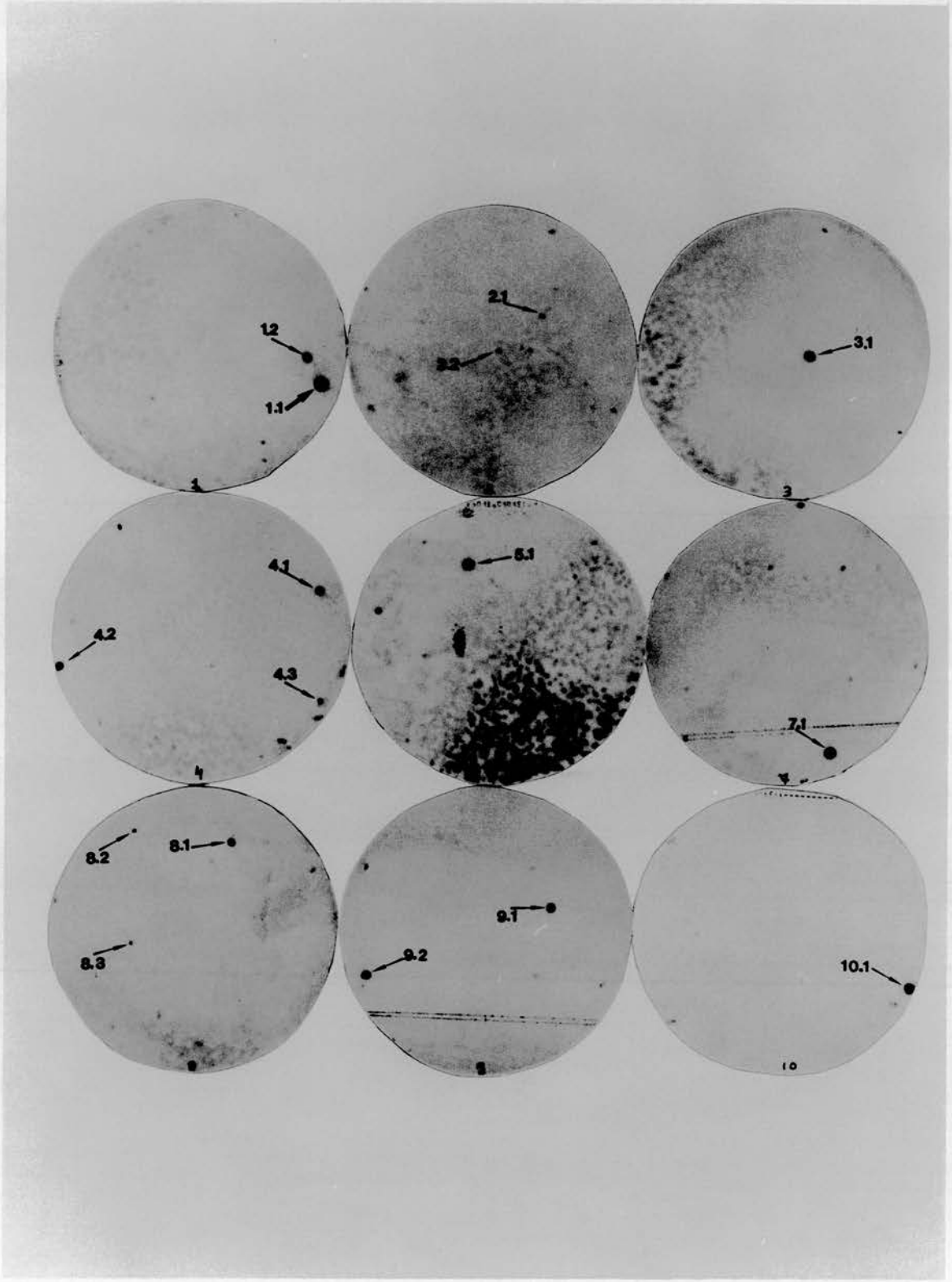
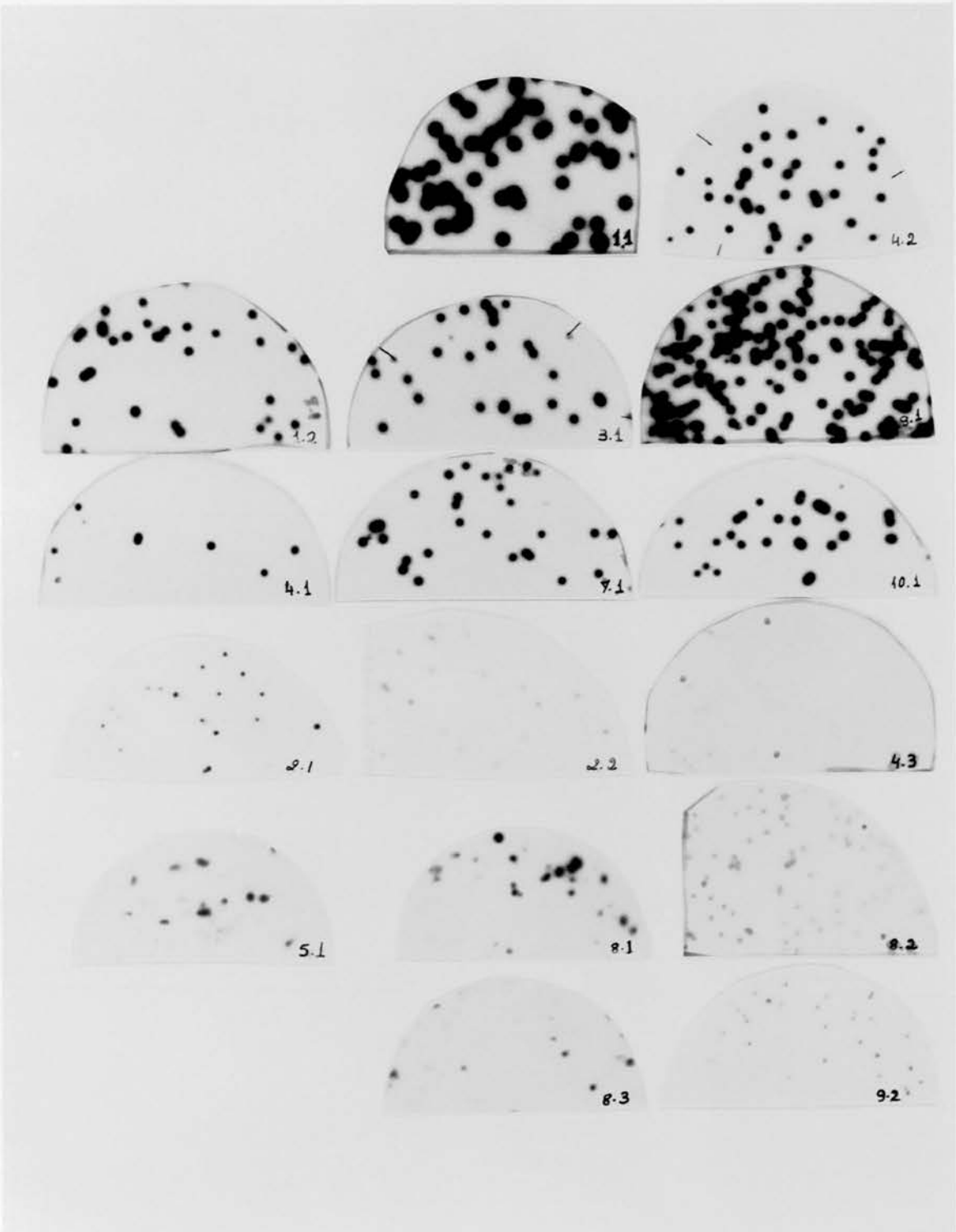

1.2
1.1
1
2.1
2.2
3.1
3
4.1
4.2
4.3
4
5.1
7.1
8.2
8.1
8.3
9.1
9.2
10.1
10

FIGURE 3.5

Second screen of the genomic ß-tubulin EcoRI clone. Autoradiography of plaque lifts from the *O. gibsoni* genomic EcoRI clones probed with *P. falciparum* ß-tubulin 0.5kb fragment (from exon II).

Hybridisation was carried out at 37°C, in 4 x SSC, 0.4mM EDTA, 0.1% NaPyrPO4, 0.2% SDS and heparin (500µg/ml). The washing was at 37°C in 0.4 x SSC and 0.1% SDS and film exposed overnight.


11
4.2
1.2
3.1
8.1
4.1
7.1
10.1
9.1
2.2
4.3
5.1
8.1
8.2
8.3
9.2

fragment. The *Plasmodium* probe hybridised with inserts from 9 clones, 5 of which appeared to have a similar size (Fig. 3.6.A, clones 1.1, 3.1, 4.1, 7.1, and 9.1).

3.5.1. Probing the Clones Against Inserts from Clones Number 1.1, 9.1 and the 39mer Oligonucleotide

To further verify the identity of the putative ß-tubulin clones they were probed with a *Plasmodium* oligonucleotide (39mer) corresponding to a peptide highly conserved in ß-tubulin from different organisms (aa 98-110: NNWAKGHYTEGA). Furthermore, to investigate the possibility that identical or overlapping clones exist amongst the population of putative ß-tubulin clones, it was decided to probe all these clones with the inserts from clones 1.1 and also 9.1, both of which gave strong signals in the original library screens (Fig. 3.6.A, B, C, D).

The inserts size and the southern blotting analysis of the putative positive clones probed with *P. falciparum* fragment, 39mer oligo, 1.1 insert, and 9.1 insert are summarised in Table 3.1.

FIGURE 3.6 (A and B)

Southern blot analysis of potential *O. gibsoni* ß-tubulin clones.

Recombinant DNA from 16 putative positive clones were digested with EcoRI enzyme, resolved, blotted and hybridised to:

A) *P. falciparum* fragment probe.

Hybridisation was carried out at 37°C, in 4 x SSC, 0.4mM EDTA, 0.1% NaPyrPO4, 0.2% SDS and heparin (500µg/ml). The washing was at 37°C in 0.4 x SSC and 0.1% SDS and film exposed overnight.

B) *P. falciparum* 39mer oligonucleotide corresponding to a peptide highly conserved in ß-tubulin from different organisms (NNWAKGHYTEGA).

Hybridisation was carried out at RT, in 6 x SSC, 0.4mM EDTA, 0.1% NaPyrPO4, 0.2% SDS and heparin (500µg/ml). The washing was at 37°C in 6 x SSC and 0.1% SDS and film exposed overnight.

1.1
1.2
2.1
2.2
3.1
4.1
4.2
4.3
5.1
7.1
8.1
8.2
8.3
9.1
9.2
10.1
23.1
9.4
6.7
4.4
2.3
2.0
A
B

FIGURE 3.6 (C and D)

Southern blot analysis of *O. gibsoni* ß-tubulin clones to investigate the possibility that identical or overlapping clones exist amongst the population of putative ß-tubulin clones.

Recombinant DNA from 16 putative positive clones were digested with EcoRI enzyme, resolved, blotted and hybridised to:

C) The 3.6kb insert from clone 1.1.

Hybridisation was carried out at 60°C in 0.5M disodium hydrogen phosphate, adjusted to pH 7.5 with 0.5M sodium dihydrogen phosphate, and 7% SDS. The washing was at 60°C in 0.2 x SSC, 0.1% SDS and film exposed overnight.

D) The 3.6kb insert from clone 9.1.

Hybridisation was carried out in very high stringency as in C.

1.1
1.2
2.1
2.2
3.1
4.1
4.2
4.3
5.1
7.1
8.1
8.2
8.3
9.1
9.2
10.1
23.1
9.4
6.7
4.4
2.3
2.0
C
D

Table 3.1. Insert size and Southern Blotting analysis

Clone no.	Insert size(kb)	Probes P.f	39mer	1.1	9.1	(+)
1.1	3.6	+	+	+	+	+
4.2	5.0	+	+	+	+	+
1.2	7.0	+	+	-	+	+
3.1	4.0	+	+	-	+	+
9.1	3.6	+	+	-	+	+
4.1	4.0	+	+	+	-	+
7.1	4.0	+	+	+	-	+
10.1	4.5	+	+	+	-	+
2.1	2.5*	(+)	-	-	-	+
2.2	6.0	-	-	-	-	-
4.3	6.0	-	-	-	-	-
5.1	3.6*	-	-	(+)	-	-
8.1	5.0*	-	(+)	-	-	-
8.2	6.0	-	-	-	-	-
8.3	2.5	-	-	-	-	-
9.2	7.5	-	-	-	-	-

* clones not included in the analysis.

The southern blot analysis of the various clones with the 4 probes showed at least three patterns of hybridisation:

1) Clones that hybridised with all 4 probes [clone 1.1 (insert size = 3.6), and clone 4.2 (insert size = 5.0)];

2) Clones that hybridised with the *Plasmodium*, 39mer oligo, and with 9.1 probes [clone 1.2 (insert size = 7.0, clone 3.1 (insert size = 4.0), and clone 9.1 (insert size = 3.6)];

3) clones that hybridised with Plasmodium, 39mer oligo and 1.1 probes [clone 4.1 (insert size = 4.0), clone 7.1 (insert size = 4.0), and clone 10.1 (insert size = 4.5)].

The pattern of hybridisation with the probes, and the wide range of insert sizes are once again consistent with the likely presence of several tubulin genes in the parasite genome. On the basis of these results it was decided to continue with the characterisation of clone 1.1 which gave a very strong positive hybridisation signal with all the probes, in particular the *Plasmodium* tubulin gene and the oligo.

3.5.2. Probing of Genomic DNA

A southern blot of *O. gibsoni* genomic DNA digested with the restriction enzyme EcoRI was probed with the 3.6kb insert derived from the EcoRI genomic clone number 1.1 (Fig. 3.7). A strong band appeared in the EcoRI digest of the expected size of 3.6kb. In addition, a 4.5kb band of significantly lower intensity was visible. The 4.5kb band may represent either a partial restriction fragment of the 3.6kb band or alternatively another gene closely related to ß-tubulin. It is interesting to note that clone 10.1 had an identical insert size of 4.5kb.

3.5.3. Restriction Enzyme Analysis of the Genomic Insert

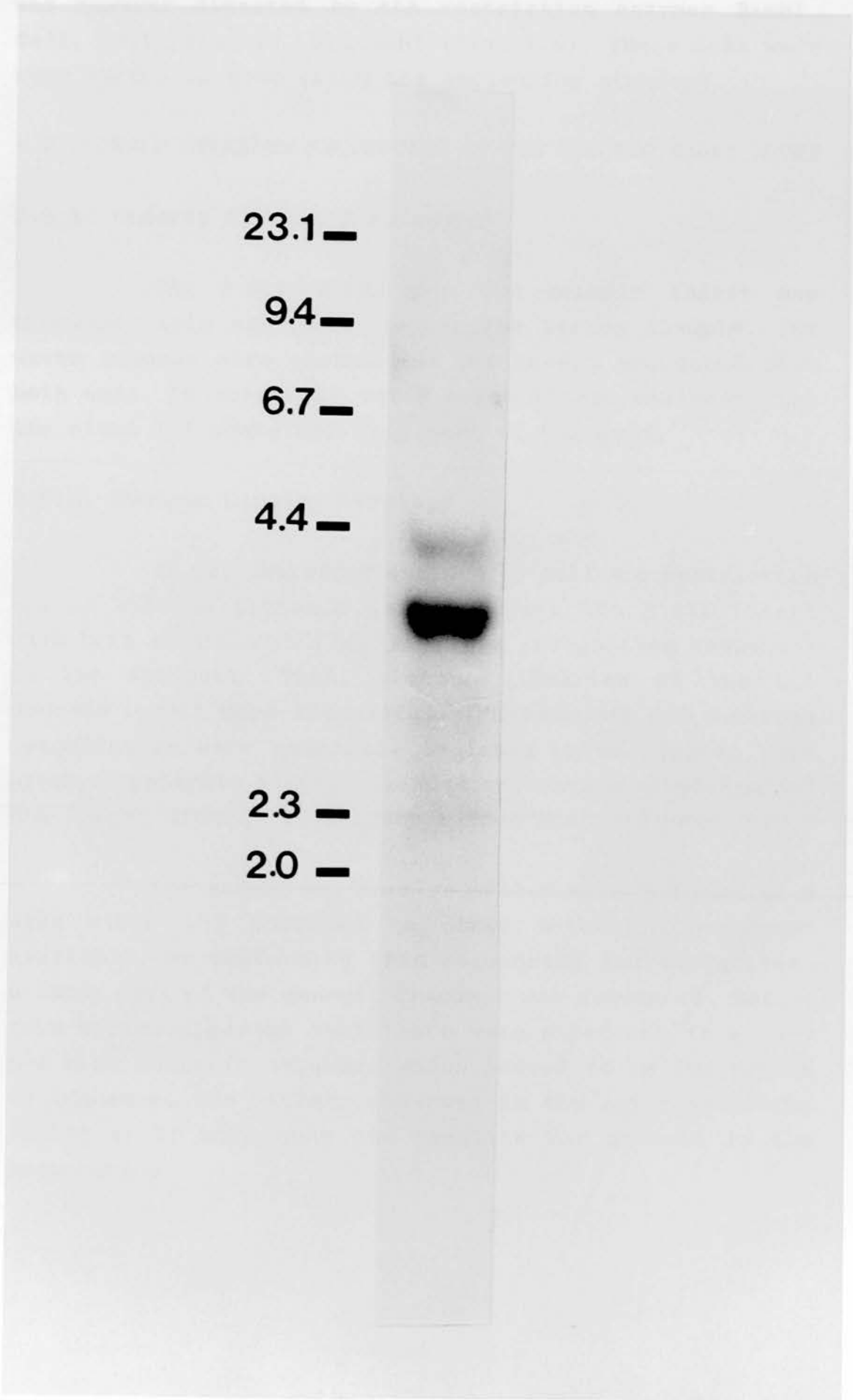
With the purpose of generating a large amount of insert for further analysis, a large scale lambda DNA preparation was performed. The DNA was digested with EcoRI in order to liberate the insert, then fractionated by agarose gel electrophoresis and purified by electroelution.

Restriction enzyme digests were performed on the purified insert. Most of the restriction enzymes tested, including BamHI, SstI, KpnI, SmaI, HpaI, XbaI, PvuII, HincII, PstI, failed to cut the genomic insert. The insert

FIGURE 3.7.

Southern blot of *O. gibsoni* genomic DNA digested with the restriction enzyme EcoRI probed with the 3.6kb insert derived from the 1.1 EcoRI genomic clone.

Hybridisation was carried out at 60°C in 0.5M disodium hydrogen phosphate, adjusted to pH 7.5 with 0.5M sodium dihydrogen phosphate, and 7% SDS. The washing was at 60°C in 0.2 x SSC, 0.1% SDS and film exposed overnight.


23.1
9.4
6.7
4.4
2.3
2.0

was however digested by the restriction enzymes BamHI, SalI, DraI, HindIII, and SphI (Fig. 3.8). These data were very useful in developing the sequencing strategy.

3.6. SINGLE STRANDED SEQUENCING OF THE GENOMIC EcoRI CLONE

3.6.1. Orderly Cloning Strategy

The 3.6kb EcoRI purified genomic insert was subcloned into the phage sequencing vector M13mp18. The white plaques were picked and the insert sequenced from both ends. At this very early stage it was realised that the clone 1.1 contained only part of the gene.

3.6.2. Shotgun Cloning Strategy

It was therefore decided to follow a restriction enzyme shotgun approach and to digest the 3.6kb insert with DraI enzyme which has multiple recognition sequences in the fragment. Thus, shotgun libraries of the 1.1 genomic insert were successfully constructed and numerous recombinants were obtained. Isolated white plaques were grown to generate single stranded sequencing templates and the Sanger dideoxy sequencing method was performed.

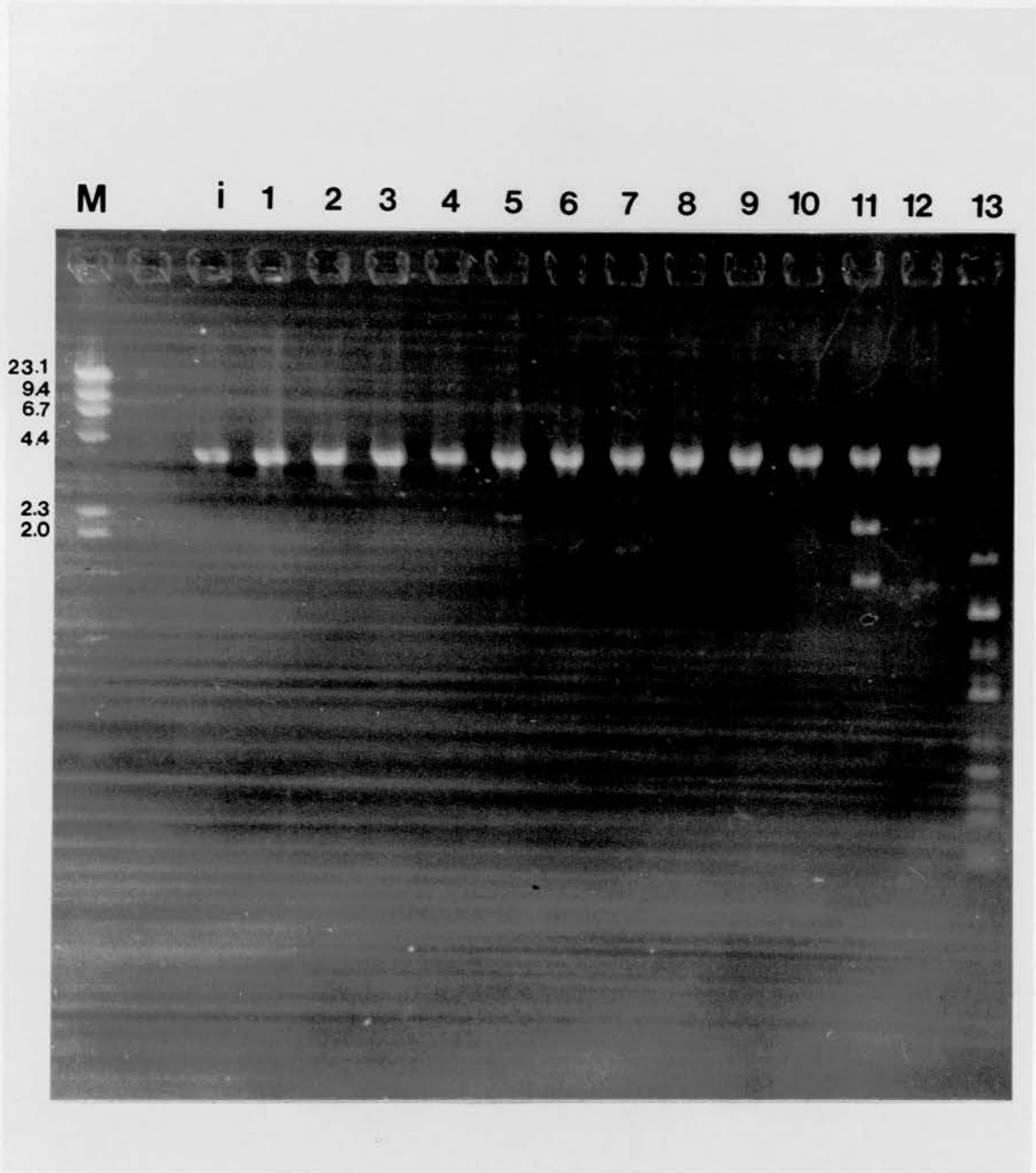
The sequences thus generated were aligned with each other and compared to other ß-tubulin sequences available. By performing this sequencing and comparison, a large part of the genomic fragment was sequenced. But it soon became apparent that there were stretches in a very A-T rich intronic sequence which proved to be impossible to sequence. The pattern observed in the autoradiography looked as if more than one template was present in the preparation.

FIGURE 3.8

Restriction enzymes digestion of 3.6kb insert from 1.1 EcoRI clone.

Ethidium bromide stained gel of purified insert DNA from 1.1 clone digested with several enzymes. Molecular weight markers (track M), uncut insert (track i). The restriction enzymes used are:

Track 1: SstI
Track 2: KpnI
Track 3: SmaI
Track 4: HpaI
Track 5: BamHI
Track 6: XbaI
Track 7: SalI
Track 8: PvuII
Track 9: HincII
Track 10: PstI
Track 11: SphI
Track 12: HindIII
Track 13: DraI


M
i
1
2
3
4
5
6
7
8
9
10
11
12
13
23.1
9.4
6.7
4.4
2.3
2.0

At this stage, it was necessary to adopt another approach to overcome the problem. The difficulty of sequencing genes containing a very A-T rich sequences had already been faced by many others (Bzik *et al.*, 1987; Snewin *et al.*, 1989; Melton, 1987b; Khan, A., personal communication; Chan, S. W., personal communication). The problem seems to be due to the instability of some foreign DNA sequences in conventional host-vector systems employed for cloning and sequencing. The common feature found in all these recombinants is the large stretch of A-T bases. According to Lauer *et al.* (1980), genes containing multiple (A-T)n ladders may give rise to secondary structures, in particular palindromes, which are targets for prokaryotic recombination systems.

Certain DNA sequences cannot be stably cloned into single-stranded M13 vectors, and such inserts are not correctly replicated and may give rise to deletions. This applies to many bacterial promoters, terminators, highly repetitive sequences and to long inverted repeats (Chen and Seeburg, 1985).

Thus, the pattern observed in the sequencing gels might be due to the instability of the large intronic sequence cloned in M13. Both clones containing deletions and clones still harbouring the full insert might be responsible for the pattern of two different templates been sequenced together. Furthermore, the generation of secondary structure, before deletion takes place, could make the sequencing difficult.

For this reason, the double stranded approach was employed using information from the restriction enzyme analysis, according to which HindIII and SphI could digest the insert clone (Fig. 3.8).

3.7. DOUBLE STRANDED SEQUENCING OF THE 1.1 GENOMIC CLONE

As the 3.6kb genomic insert and some of its subfragments were unstable in single stranded M13 sequencing vector, it was decided to employ the technique of double stranded sequencing so that the sequencing could be completed.

In order to access sequence at the 5' end of this intervening sequence and to fill the gaps from the shotgun strategy, a set of subclonings were performed using the pUBS plasmid (Fig. 2.3; section 2.2) as the cloning vector.

Restriction digestion of the insert with HindIII and/or SphI provided a number of fragments that could be subcloned in pUBS in the following way: 1) HindIII/HindIII fragments for ligation in HindIII digested pUBS; 2) EcoRI/HindIII fragments used in a forced ligation in EcoRI/HindIII digested pUBS; 3) EcoRI/SphI fragments again in a forced ligation in EcoRI/SphI digested pUBS. The ligations were transformed into JM83 cells, and the positive recombinants selected by hybridisation with the 3.6kb insert from clone 1.1 after colony lifts were taken.

This strategy proved to be very successful, generating several recombinants which were isolated and grown to provide good DNA for the double stranded sequencing. The design of 4 oligonucleotides (#0, #1, #4, and #5; section 2.2.26.4.1) was also necessary to complete the sequencing. The total sequence, derived from both the single and double stranded sequencing is compiled in figure 3.16, and the sequencing strategy is summarised in Fig. 3.9.

The DNA and deduced amino acid sequences will be

analyzed in detail later in this chapter.

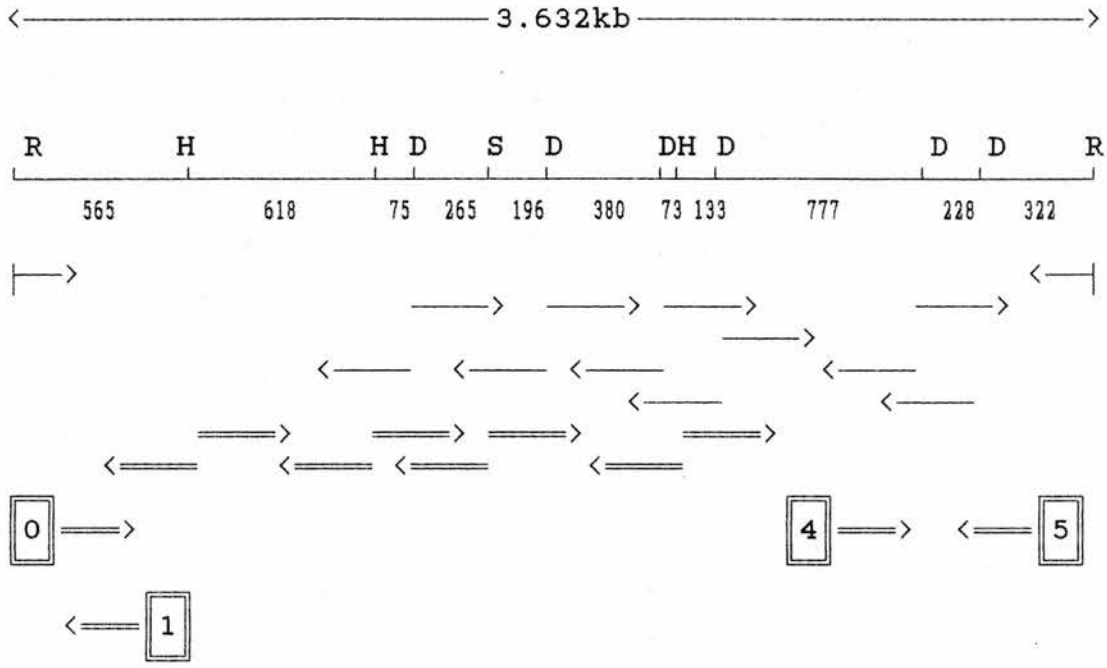


Fig. 3.9. Restriction map and sequencing strategy of the 3.6kb insert EcoRI clone containing part of ß-tubulin gene of *O. gibsoni*. Arrows indicate the extent and direction of the sequencing. The single line arrows show the single-stranded sequencing in M13 and the double line ones represent double-stranded sequencing in plasmid (pUBS). Numbers within boxes correspond to oligonucleotides designed to complete the sequencing in the double-stranded cloning λNM1149 vector. Only restriction sites utilised in sequencing are shown. Enzyme abbreviations are: R, EcoRI; H, HindIII; S, SphI; D, DraI.

3.8. ISOLATION OF OVERLAPPING 5'- AND 3'-END CLONES.

The sequence derived from clone 1.1 was limited to a large central portion of the ß-tubulin gene. Thus, the entire gene was not contained within the 3.6kb EcoRI clone insert. As there are conveniently placed Hind III sites in close proximity to the distal 5'- and 3'-ends of

this cloned insert (Fig. 3.9), it was decided to screen the *O gibsoni* Hind III genomic DNA library to isolate overlapping clones. Thus, the 0.565kb and 1.4kb EcoRI/HindIII restriction fragments from clone 1.1 would appear to be ideal probes. In practice, however, even after gel purification of these fragments there would inevitably be some, though minor, contamination with unwanted insert and vector fragments which could complicate the library screens.

As there are two appropriate pairs of sequencing primers contained within these two EcoRI-HindIII regions (Fig. 3.9), it was decided to circumvent this problem by enzymatically amplifying the fragments bounded by the primers using the polymerase chain reaction (PCR). Thus, the PCR technique (section 2.2.24 in chapter 2) was employed to provide amplification of DNA sequences to be used as probes for the screening of a HindIII library.

3.9. AMPLIFICATION OF DNA FRAGMENTS (PCR) TO BE USED AS PROBES.

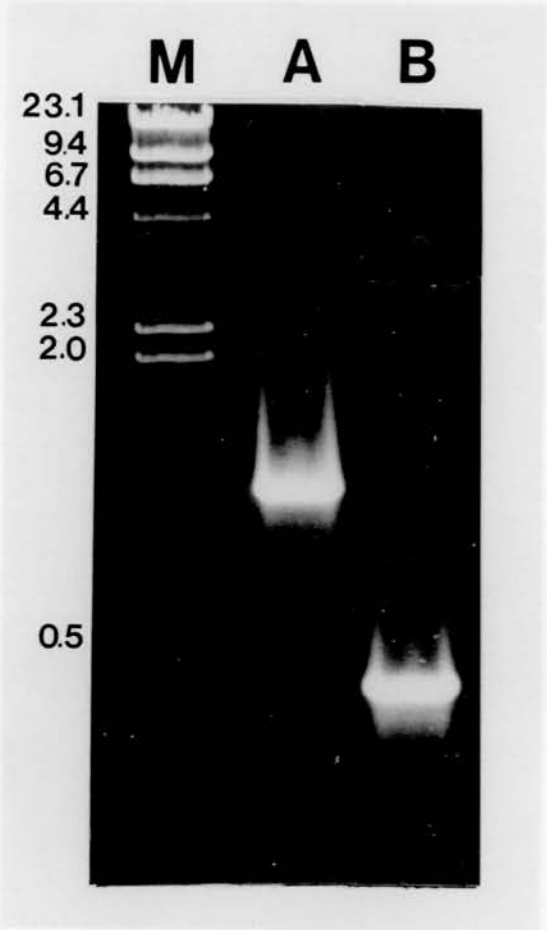
The polymerase chain reaction (PCR) is a novel technique that amplifies specific sequences with remarkable efficiency. Repeated cycles of denaturation, primer annealing and extension carried out with the heat stable enzyme, a Taq polymerase, leads to exponential increases in the target DNA sequences.

Thus, using clone 1.1 as the template DNA, a 5'-end probe of 320bp was generated with oligonucleotides 0 and 1, and a 3'-end probe of 1.2kb produced with oligonucleotides 4 and 5 (Fig. 3.10.A and B). The PCR products were gel purified, radiolabelled, and used as probes to screen the Hind III genomic library.

FIGURE 3.10.A

Ethidium bromide stained gel of PCR amplification products.

Track M: λ DNA markers
Track A: PCR 2 product (1.2kb)
Track B: PCR 1 product (0.320kb)


M
A
B
23.1
9.4
6.7
4.4
2.3
2.0
0.5

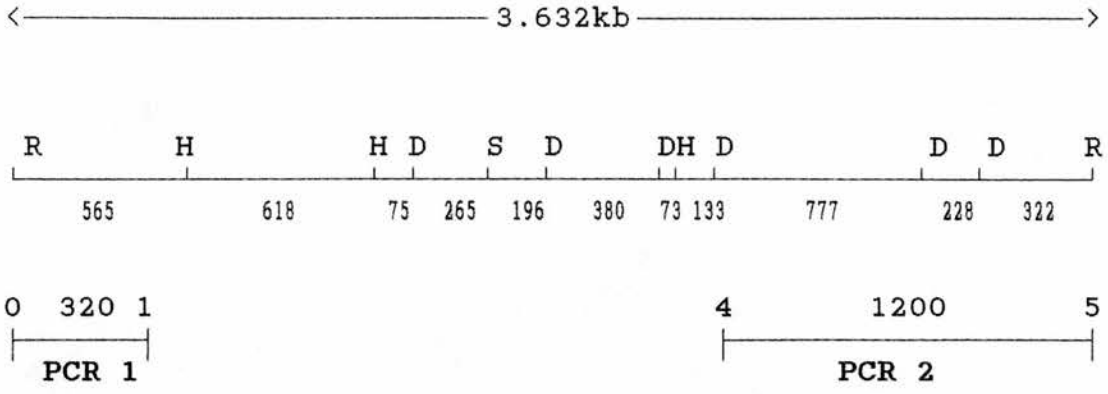


Fig. 3.10.B. Restriction map of clone 1.1 insert. The PCR 1 and PCR 2 are the sequences, bounded by the primers (0/1 and 4/5 respectively), enzymatically amplified by PCR. PCR1 and PCR2 represent the fragments utilised as probes to isolate the recombinants containing the N-terminal (PCR1) and the C-terminal (PCR2) regions of the ß-tubulin gene from *O. gibsoni*. Enzyme abbreviations are: R, EcoRI; H, HindIII; S, SphI; D, DraI.

Thus, a library constructed from HindIII digested *O. gibsoni* genomic DNA was screened with the PCR1 and PCR2 products for the N-terminal (PCR1) and the C-terminal (PCR2) regions of the ß-tubulin gene. The HindIII library was plated out at a concentration of 1.2×10^3 recombinant phages per plate and duplicate plate lifts were taken. The hybridisation was performed at high stringency in presence of Na_2HPO_4 in 7% SDS, at 60°C (section 2.2.21.1) and the labelled probe (PCR1 or PCR2). After hybridisation, the filters were washed in 0.1% SDS, 0.2 x SSC at 60°C for 2 hrs. Following this procedure it was possible to isolate both the N-terminal and the C-terminal clones, and once again the number of putative positive clones in the first screening (Fig. 3.11.1) was considerably large. But just the clones presenting stronger signals (clone N-t, and clone C-t) were picked, enriched and purified (Fig. 3.11.2).

FIGURE 3.11 (1,2 and 3)

Isolation of the genomic ß-tubulin HindIII clone.

1) Autoradiography of plaque lifts from the first screening of the *O. gibsoni* genomic HindIII library probed with PCR1 (on the left → N-terminal clone) and PCR2 products (on the right → C-terminal clone).

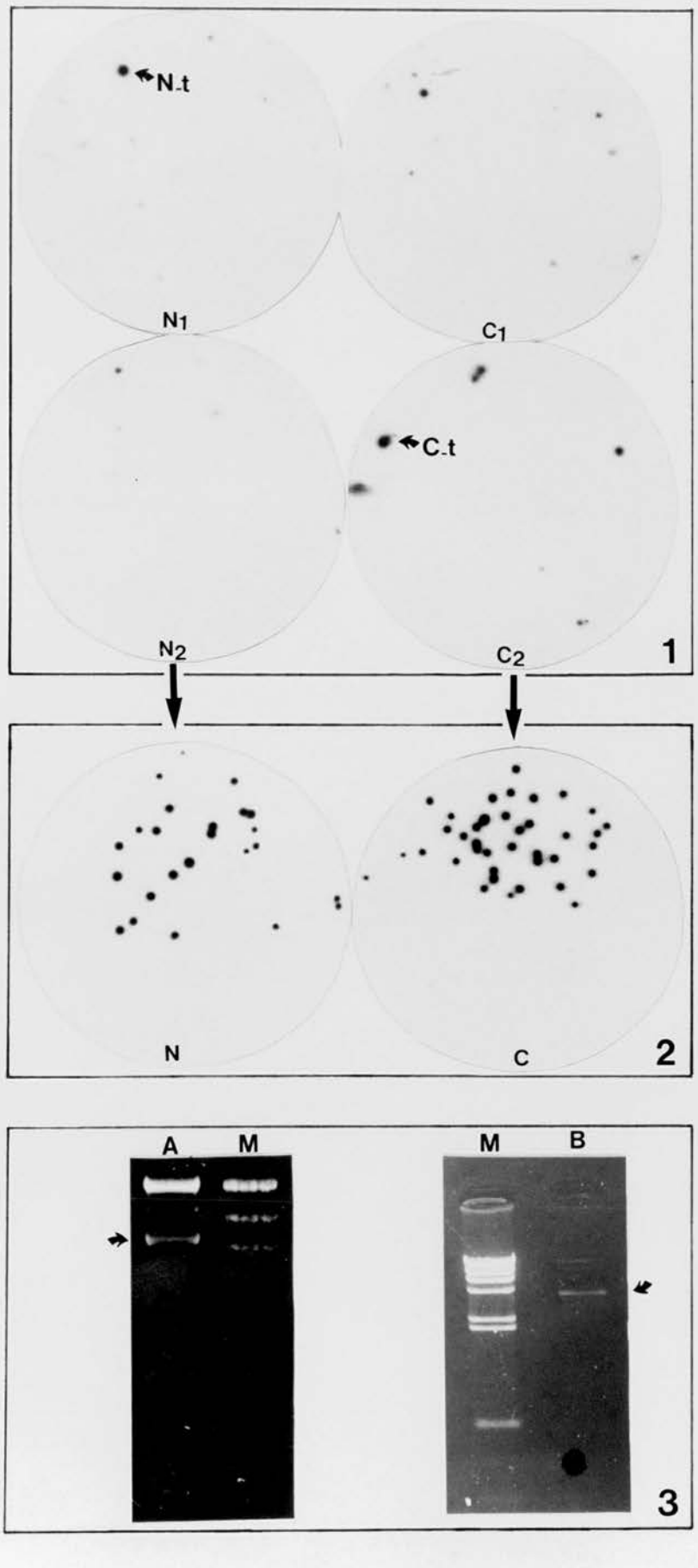
Hybridisation was carried out at 60°C in 0.5M disodium hydrogen phosphate, adjusted to pH 7.5 with 0.5M sodium dihydrogen phosphate, and 7% SDS. The washing was at 60°C in 0.2 x SSC, 0.1% SDS and film exposed overnight.

2) Autoradiography of plaque lifts from the second screening of the *O. gibsoni* genomic HindIII library probed with PCR1 (on the left → N-terminal clone) and PCR2 products (on the right → C-terminal clone).

Hybridisation was carried out at 60°C in 0.5M disodium hydrogen phosphate, adjusted to pH 7.5 with 0.5M sodium dihydrogen phosphate, and 7% SDS. The washing was at 60°C in 0.2 x SSC, 0.1% SDS and film exposed overnight.

3) Determination of insert size from the λNM1149 N-terminal HindIII clone (on the left) and C-terminal HindIII clone (on the right).

Ethidium bromide stained gel of HindIII digested phage DNA from the N-t clone (on the left), and C-t clone (on the right). Arrows show the insert size for N-t clone at 7.0kb and for C-t clone at 4.0kb.


N.t
N1
C1
C.t
N2
C2
1
N
C
2
A
M
M
B
3

3.9.1. Determination of insert size from both N-t and C-t Clones.

DNA from both clones (clone N-t and C-t) were prepared, digested with the restriction enzyme Hind III to liberate the inserts and fractionated by agarose gel electrophoresis. The insert sizes were 7.0kb and 4.0kb for the N-terminal clone and C-terminal clone respectively (Fig. 3.11.3).

3.9.2. Sequence Determination of the 5'-end Clone

To further characterise the 5'-end clone N-t it was decided to apply the double-stranded DNA sequencing technology to the lambda clones. This proved to be very successful. Sequencing of the 5'-clone primed with oligonucleotide 1 generated sequence which precisely matched the sequence originally derived from clone 1.1, hence rapidly confirming the identity of the clone without further subcloning.

To generate new sequence from this clone a primer (oligonucleotide A) was tailored to match sequence in close proximity of the 5' boundary of clone 1.1. Surprisingly, oligo A generated only a limited amount of sequence (approximately 70bp) which immediately terminated after a stretch of 13 T residues within the intron. A primer, oligonucleotide A2, was designed from the sequence immediately after the run of T residues. Unfortunately, this primer also failed to generate sequence past the stretch of T residues.

To overcome this problem, a drastically different strategy was adopted. Oligo A and also a heterologous oligo (38 mer) based on the 5'-end of the *Plasmodium* ß-tubulin gene beginning with the starting codon were used as primers in an attempt to amplify the

sequences they bounded from the clone N-t. The outcome of this experiment critically depended upon two factors. Firstly, the 5'-ends of the ß-tubulin genes are sufficiently conserved between the two organisms in a way such that the *Plasmodium* 38mer had a sufficient amount of matches with the *O. gibsoni* gene to anneal in a thermodynamically favourable manner. Secondly, an appropriate number of the 5' terminal bases of the *Plasmodium* 38mer perfectly matched to the target sequence so as to allow the enzyme Taq DNA polymerase to extend the primer.

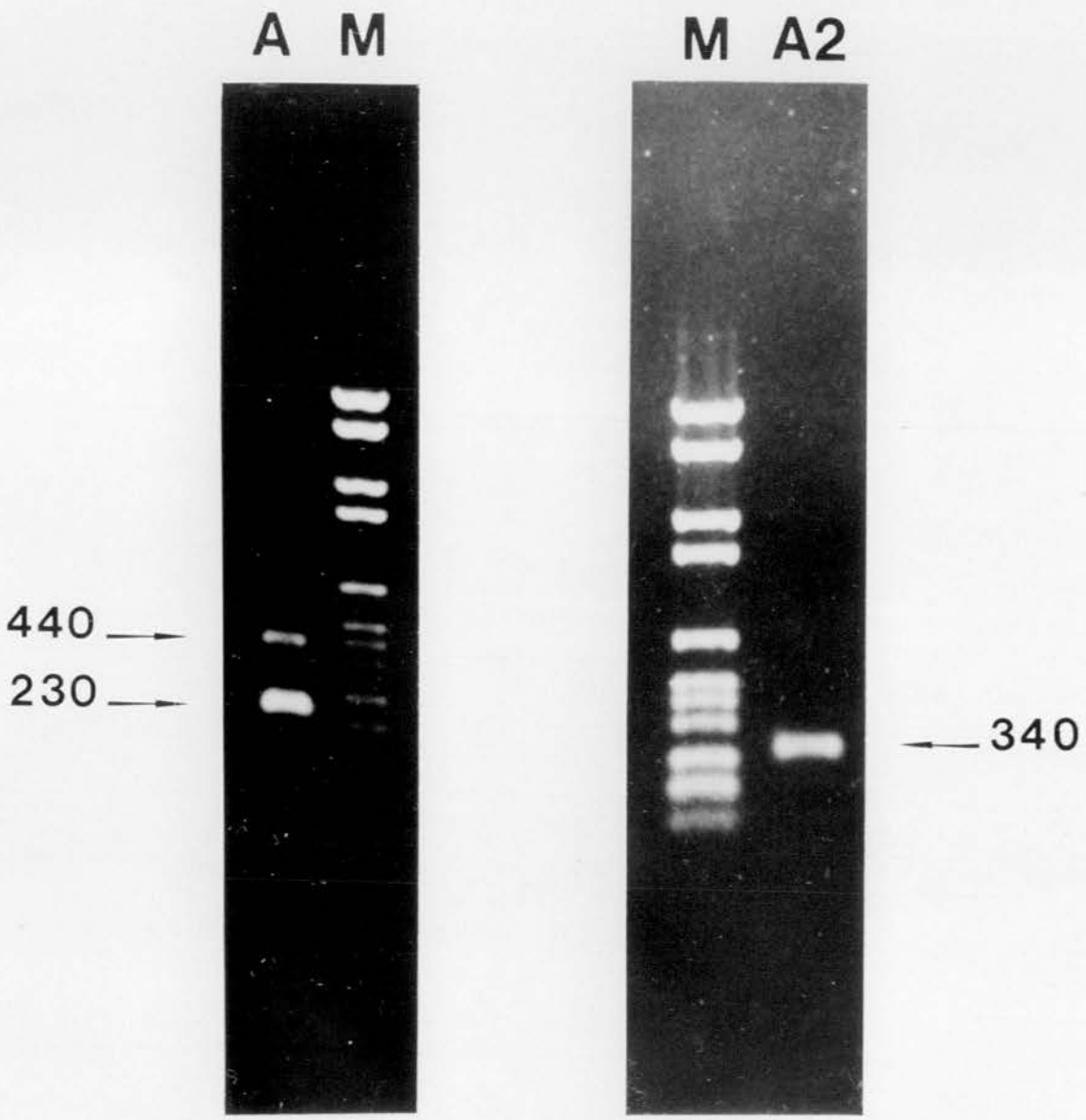
Remarkably, despite these potential technical barriers the PCR was successful. Two products were amplified from the template DNA (clone N-t) of size 440bp and 230bp (Fig. 3.12). The fragments were gel purified and each again subjected to PCR this time replacing oligo A with an internal primer oligo A2. Only the 440bp fragment gave rise to a 340bp PCR product, suggesting the initial 440bp PCR product to be the desired fragment. The PCR fragment produced with oligo A2 and the *Plasmodium* 38mer was gel purified, kinased, and ligated into the Sma I site of M13mp19. Following transformation, white plaques were picked and single-stranded template DNA generated. The insert was completely sequenced with universal primer and gave rise to sense-strand sequence which overlapped and extended the existing sequence through to the *Plasmodium* 38mer sequence.

Surprisingly, another stretch of 20 T residues was present in the clone. This probably would be another barrier to the double-stranded sequencing. However, such residues were now a stretch of A residues in the M13mp19 single-stranded template, and created no sequencing problems. Another fact to be considered is that the small insert (340bp) was probably more stable in the cloning vector.

FIGURE 3.12

Ethidium bromide stained gel of PCR amplification products.

Track M: DNA molecular weight markers (pBR 328 BglI digest plus pBR 328 HinfI digest)
Track A: primary PCR
Track A2: secondary PCR


A M
M A2
440
230
340

However, as the *Plasmodium* 38mer imposed its own sequence on the *O. gibsoni* gene, the true sequence was determined by designing a primer (oligo B) based on the M13mp19 sequence after the stretch of T residues in the lambda clone N-t. This approach successfully yielded sequence up to and beyond the assumed starting codon (Fig. 3.16). The sequencing strategy for the 5'-end of the Hind III clone is shown in Fig. 3.13, and the position of all the oligonucleotides used in this strategy is shown in Fig. 3.14.

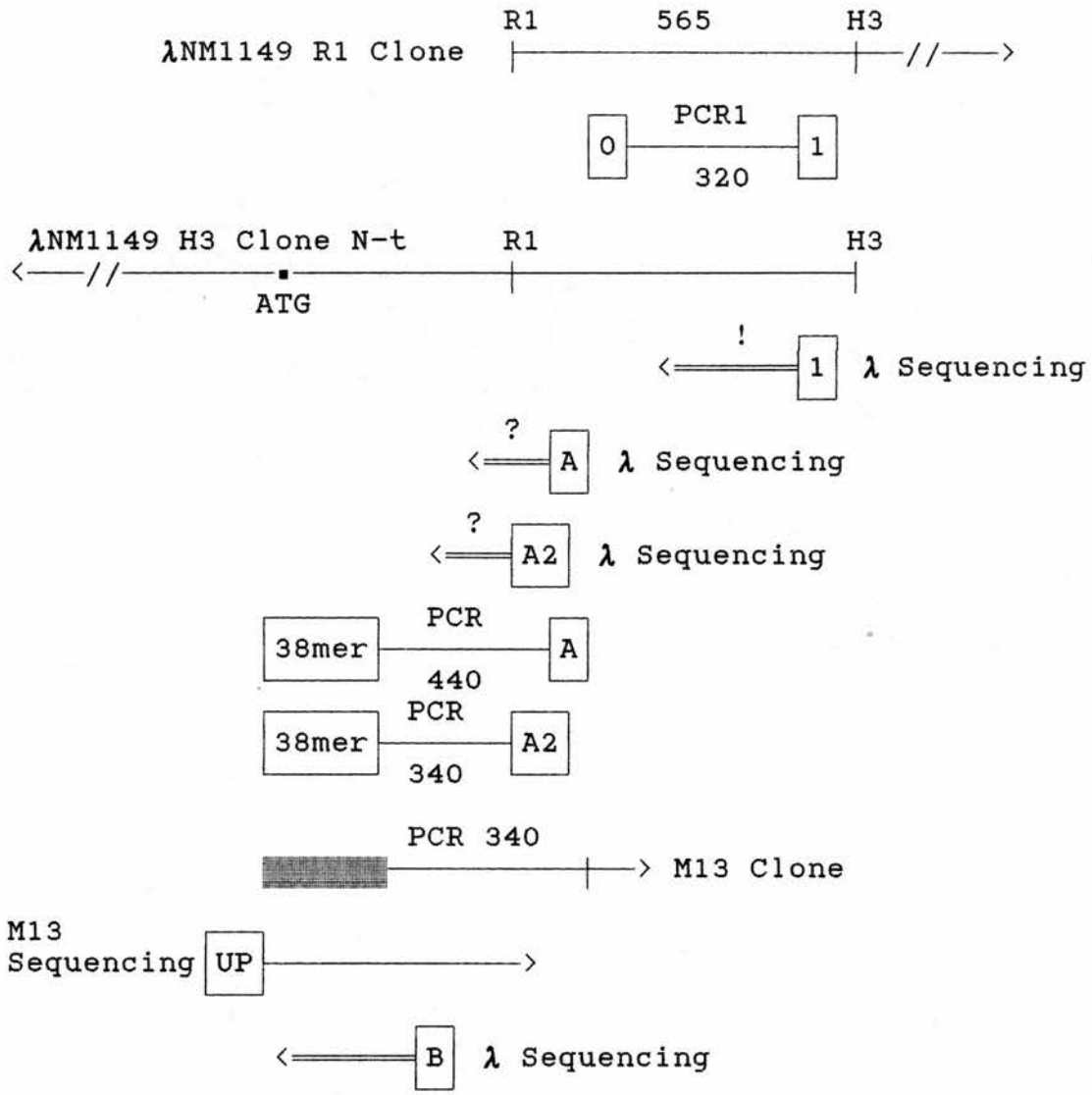


Fig. 3.13. Restriction maps and sequencing strategy of the 5'-end HindIII clone of ß-tubulin gene from *O. gibsoni*. On the highest resolution map only part of the original λNM1149 EcoRI clone (1.1) is indicated. The important restriction sites in the generation of the PCR1 probe are: R1, EcoRI; and H3, for HindIII. Numbers and letters in the boxes correspond to the oligo nucleotides designed for the completion of the sequence (UP oligo is the universal primer). Single-stranded sequencing strategy (M13) is indicated by single arrows and double-stranded sequencing (in λNM1149) by double arrows. The grey box represents the *P. falciparum* 38mer sequence imposed on the *O. gibsoni* gene fragment. '!' and '?' stand for successful and unsuccessful sequencing respectively.

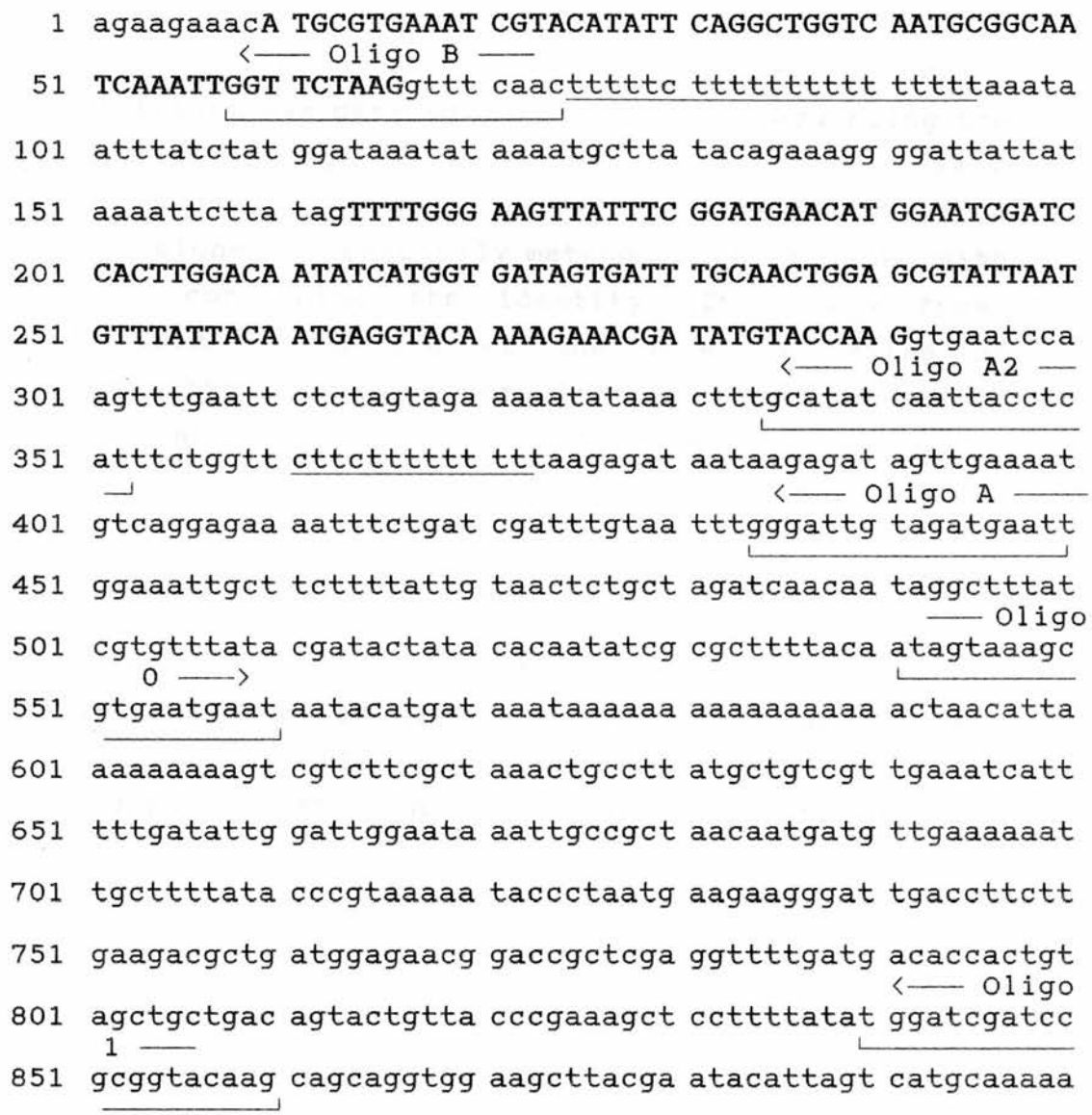


Fig. 3.14. 5'-End of the ß-tubulin gene from *O. gibsoni*. The sequence shows the position of the oligonucleotides utilised to generate the PCR1 probe (oligos 0 and 1). It also shows the position of oligos A, A2, and B, which were utilised to overcome the problem with the sequencing of the stretches of Ts (both underlined).

3.10. SEQUENCE DETERMINATION OF THE 3'-END CLONE

The identity of the 3'-end clone C-t harbouring the 4.0kb insert was determined by directly sequencing the lambda DNA with oligo 4, which was initially used as a primer for clone 1.1 (Fig. 3.9). The sequence derived from the 3'-end clone C-t precisely matched that obtained with clone 1.1, confirming its identity. To access fresh sequence from the 3'-end of the gene, a series of successively overlapping oligonucleotides were synthesised (Fig. 3.15) and the sequence of the *O. gibsoni* ß-tubulin gene completely determined from 3 recombinants: clone 1.1 EcoRI, clone N-t HindIII, and clone C-t HindIII (Fig. 3.15).

This strategy proved to be very successful, generating the necessary data to cover the ß-tubulin gene from *O. gibsoni* and showing that it comprises a total of 5,909 base pairs containing 12 exons and 11 introns (Fig. 3.16 and 3.17).

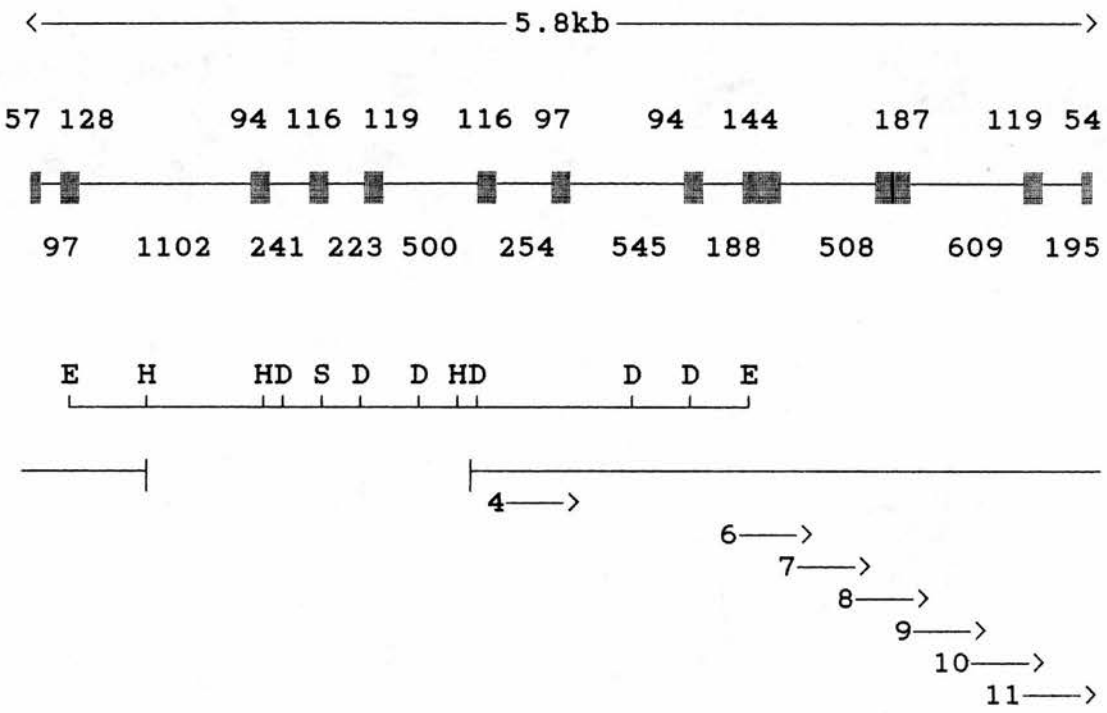


Fig. 3.15. Structure and organisation of ß-tubulin gene from *O. gibsoni* and the 3 clones needed to cover gene: HindIII-clone N-t, EcoRI-clone 1.1, and HindIII-clone C-t. The arrows with numbers in HindIII-clone C-t diagram represent the sequencing strategy and the series of the successively overlapping oligonucleotides employed.

Figure 3.16. Nucleotide Sequence of *O. gibsoni* ß-Tubulin Gene

```
   1  agaagaaacATGCGTGAAATCGTACATATTCAGGCTGGTCAATGCGGCAA
                 M  R  E  I  V  H  I  Q  A  G  Q  C  G  N   14
  51  TCAAATTGGTTCTAAGgtttcaacttttctttttttttttttttaaata
       Q  I  G  S  K                                       19
 101  atttatctatggataaatataaaatgcttatacagaaaggggattattat
 151  aaaattcttatagTTTTGGGAAGTTATTTCGGATGAACATGGAATCGATC
                      F  W  E  V  I  S  D  E  H  G  I  D   31
 201  CACTTGGACAATATCATGGTGATAGTGATTTGCAACTGGAGCGTATTAAT
      P  L  G  Q  Y  H  G  D  S  D  L  Q  L  E  R  I  N   48
 251  GTTTATTACAATGAGGTACAAAAGAAACGATATGTACCAAGgtgaatcca
       V  Y  Y  N  E  V  Q  K  K  R  Y  V  P  R           62
 301  agtttgaattctctagtagaaaaatataaactttgcatatcaattacctc
 351  atttctggttcttctttttttttaagagataataagagatagttgaaaat
 401  gtcaggagaaaatttctgatcgatttgtaatttgggattgtagatgaatt
 451  ggaaattgcttcttttattgtaactctgctagatcaacaataggctttat
 501  cgtgtttatacgatactatacacaatatcgcgcttttacaatagtaaagc
 551  gtgaatgaataatacatgataaataaaaaaaaaaaaaaaaactaacatta
 601  aaaaaaaagtcgtcttcgctaaactgccttatgctgtcgttgaaatcatt
 651  tttgatattggattggaataaattgccgctaacaatgatgttgaaaaaat
 701  tgcttttatacccgtaaaaataccctaatgaagaagggattgaccttctt
 751  gaagacgctgatggagaacggaccgctcgaggttttgatgacaccactgt
 801  agctgctgacagtactgttacccgaaagctccttttatatggatcgatcc
 851  gcggtacaagcagcaggtggaagcttacgaatacattagtcatgcaaaaa
 901  aaattggataaatgaggattaaagatgaacaattctcaacaacatttcac
 951  acgatgcataagctctattagcaaatcgaaagatgagaaaatgaagcctt
1001  tgcgtcaatatttaggaaagaaattcatttgagaaatattacaaattatg
1051  ctagaaataatattataaatatcacaaaatacaaatacttcgattattgc
1101  attttatttatattatgaatatttcggttttattgacagaagtaataaat
1151  ttttagaaaaaaaaaaaaaaaaaaaagcaactactataaattgtaaaatt
1201  gtaagaatatttaactggaacttgtaaaaatattttactgaaaagtttgt
1251  taaaatatttggctgattctcaaaactgctatgaacagtaaggatatttg
1301  actgggtaggcttttccggaattttccataatggcaaataagtttccttg
1351  ttttttttttttttttttttcttgtaattcgaacaaatttcagAGCAATT
```

```
                                                          A  I    64
1401  TTGGTAGATTTGGAACCGGGAACAATGGATAGTGTACGAGCTGGTGCTTT
       L  V  D  L  E  P  G  T  M  D  S  V  R  A  G  A  F   81
1451  CGGACAGCTTTTTCGACCCGATAATTATGTTTTCGgtgaagctttttttt
        G  Q  L  F  R  P  D  N  Y  V  F                    92
1501  ttttccactgataaaattcaaaataaataataagttcgtttgaaattgac
1551  gaatttaattctttaaaacatagcgataattaattgccaagcgtttgtta
1601  taatgaagtaattctgctaatttatattatttttttattatcatttctttc
1651  atatctaaatttcttttcataagacaaaattagtgcattttatttttttct
1701  cgttaataatacagaaatatttccagGTCAAAGTGGAGCTGGTAATAACT
                                  G  Q  S  G  A  G  N  N   100
1751  GGGCCAAAGGTCATTACACTGAAGGAGCTGAATTGGTTGATAGTGTATTG
      W  A  K  G  H  Y  T  E  G  A  E  L  V  D  S  V  L   117
1801  GATGTGATTCGTAAAGAGGCTGAAGCATGCGATTGCTTACAAgtaatatc
       D  V  I  R  K  E  A  E  A  C  D  C  L  Q           131
1851  attttcagaaaaaaaagatgatgaaatatttaatgaagaattattaattc
1901  aaaaaaaagaaaagagaaagtgagaaattgaaaatgcaacttattacttt
1951  tagtaattaattatttaagcaaatttatttcaaatactgaaaatttgtgg
2001  aattgtggaaatatttgtatactttaaattatgcatactgtatactgata
2051  tattatttcatttagGGTTTTCAATTTACTCATTCATTGGGAGGTGGTAC
                        G  F  Q  F  T  H  S  L  G  G  G  T  143
2101  TGGTTCCGGTATGGGTACATTATTGATATCGAAAATTCGTGAAGAATATC
        G  S  G  M  G  T  L  L  I  S  K  I  R  E  E  Y   159
2151  CGGATCGTATTATGACTACCTTTTCGGTGGTTCCTTCGCCGAAGgtatac
      P  D  R  I  M  T  T  F  S  V  V  P  S  P  K        174
2201  taatttattgataatcaattctattaaatgacgaaaaactatgtcggatc
2251  atttgaattatttcaaaatcataaattttgattataattttatgattaca
2301  ttagaaatttatgtctggagttgtggaaaattgcgagaattgttaattat
2351  ttcgtcttatttcgattctttcgtttacatttgaatttattgagaagaaa
2401  tgtttaaaatcaaattgaacaagcaatttgttaaaagattaaaaaaagat
2451  tagagtagactttaagttagcaaaaataagcttcagctacttaaaaatta
2501  tcaagaaacattaagtttgtatgaaatgaaaattatattctcgggccaac
2551  taaatttgcaacgagaaaaaaaaactgatgaataatataaaaatcttatg
2601  tataattatttaaatgatattattagaagtaatgaaaatatcaaaataaa
2651  tatttcgaagttttttttttttcttctttgtaataatttcagGTATCG
                                                    V  S   176
2701  GATACAGTAGTTGAACCATACAATGCCACATTATCGGTGCATCAATTGGT
```

```
       D  T  V  V  E  P  Y  N  A  T  L  S  V  H  Q  L  V  193
2751   TGAAAATACCGACGAAACGTTTTGTATTGATAATGAAGCATTATACGATA
         E  N  T  D  E  T  F  C  I  D  N  E  A  L  Y  D    209
2801   TCTGTTTCAGgtatcacttttttatcagatacttgaagtaattgacagta
       I  C  F  R                                          213
2851   accgtgatataactttgaaatatacatatagattgcttatcagttaatag
2901   ctatgaatcttattcagaaatgcttggagaatatcattcagaaactttcc
2951   ttgaattgtcgtgcattttagtctgaaatattctcaattctcatgtattt
3001   catgtatcttcacatcacaagaaaatgaaagtattataatgatttatctt
3051   aacaaaaattgcagAACACTGAAATTGACAACACCGACGTATGGCGATCT
                           T  L  K  L  T  T  P  T  Y  G  D  L  225
3101   GAATCATTTGGTTAGTGCAACAATGAGCGGTGTCACGACTTGTTTAAGAT
         N  H  L  V  S  A  T  M  S  G  V  T  T  C  L  R    241
3151   TTCCGGGACAAgtttgtttcttccattcttttattattattattattatt
       F  P  G  Q                                          245
3201   attattattattattattattattattattattatttgaattatgca
3251   gctaaaagtcggtggaatgatgatttcatgaaggaaacttgaaaatgatt
3301   tatgactaatgtttatcttttatttagctattttgtgaaaaaattttaat
3351   atttcgattttattaaaataaatgcatttttatatttaaaattgttaat
3401   taacaagtataatgccaataaataaatgttaataattccaaatttatgga
3451   agcaaaatatttggtctcaaaatcgttaaatgtgaaaaatcattaatttc
3501   atccagatctttaattgcttataatttcctaattctaaaagatatgaga
3551   tatttgtcatactgttcaaatttgatgcaactaaactattaaataggatc
3601   attacttttaatttaaatttacttgaattaaaatcaaataattaaaa
3651   aatatagaggtatttatattaatttgaaaaaattgtcttgtctcaaaata
3701   ttttagTTAAATGCTGATTTACGCAAACTGGCTGTTAATATGGTACCATT
                 L  N  A  D  L  R  K  L  A  V  N  M  V  P  F  260
3751   TCCGCGATTGCACTTTTTCATGCCCGGTTTTGCACCACTCACTTCTCGAA
         P  R  L  H  F  F  M  P  G  F  A  P  L  T  S  R    276
3801   gtttgtttttttttttctttccttttttatttataaactttggtttgtaaa
3851   aaaatgaataaataatatattacaaaatctatcaggctaataattttgat
3901   taatagaagatattgattggagcacacttggaatatagaattcataattc
3951   ataaaagtcgaaaataaacttattcaaatttttgatagGTAATCAACAG
                                             S  N  Q  Q  280
4001   TATCGTGCTGTTACGGTAGCTGAATTAACTCAACAATTATTTGATGCTAA
         Y  R  A  V  T  V  A  E  L  T  Q  Q  L  F  D  A  K  297
4051   AAATATGATGGCTGCTTGCGATCCGAGGCATGGACGTTATCTTACAGCCG
```

```
          N  M  M  A  A  C  D  P  R  H  G  R  Y  L  T  A    313
4101  CTGCAATTTTTCGTGGAAGAATGTCAATGAAGgtttctatctttctattt
      A  A  I  F  R  G  R  M  S  M  K                       324
4151  ctttttttttttttttttaacttcaattcaaacttatgcataagatttc
4201  ttatattacgttaatttgtttgttgttaaaaggatttcttataatgaatc
4251  aaaattacaaaattaatagatatacttagactgtcttaaatgaatcattt
4301  gaaatttcaactaacttcatttttcttaaattaattttatcatgtatcta
4351  atgaaatatatttcagctagaatttaaattctgtattataaaactaccaa
4401  cgaatcgaaattagcgttttctgcttattttcatcaatttagttaattta
4451  acgatgactgttcaacacaagcgttgattacatgaaataattcaacatgc
4501  gcatatagtgcagttgtgtcgtctcctagtaaaagcaaccaacaccattc
4551  cggaaacaaaaaaaaagaaaaaagttgaatgttcaaaatcgaaatataaa
4601  atatgtttacatgaatgaatgataactttactcgtttaagGATGTTGATG
                                                D  V  D    327
4651  AACAAATGTTGAACATACAAAATAAAAATTCCGCATATTTCGTCGATTGG
      E  Q  M  L  N  I  Q  N  K  N  S  A  Y  F  V  D  W    344
4701  ATTCCAAATAATGTGAAAACAGCCGTATGCGATATACCACCACGTGACCT
       I  P  N  N  V  K  T  A  V  C  D  I  P  P  R  D  L   361
4751  CAAAATGGCTGCAACATTTATTGGCAATTCTACGGCAATCCAAGAACTTT
        K  M  A  A  T  F  I  G  N  S  T  A  I  Q  E  L     377
4801  TCAAACGTGTTTCCGAACAGTTCACGGgttaattttatcaaatttattcg
      F  K  R  V  S  E  Q  F  T                             386
4851  attgattagtatgagatattcaaggatgatttcatctatgtaaatatgtt
4901  cgttgtggttcttcatacagtgttacatttgagtaggcgcgtatttttat
4951  atatagatacctactttttctatctgcgcacaaggcaaacatgtttccc
5001  gccaaaacgctccgattaaaagataaacaaagtgatatcatcaatcaatt
5051  attgattcactgctagataatggtggatcaataatttatataaatagaaa
5101  tgtaaatgttcgttatggtttttaacacagctttcaagttcttcatcga
5151  ttgatttgaatttctgaaacaaagttgccattcacatagatgcttctttt
5201  ttttacctgtcacaccttgtgatgggtaaaagtaagtgagtggatatttt
5251  catttatatagaccaacggccggccagtgcttcaagtggccggatttcag
5301  ctggttactttatgaaaatatgttgaaatatgtaattgtagaattgagaa
5351  attactggatctttttacgtaaaatttaaaatcttatgtaaaataaggt
5401  atttacatcattcgtactataatgacaatattttagCGATGTTTCGACGG
                                          A  M  F  R  R    391
5451  AAAGCATTTCTGCATTGGTATACCGGTGAAGGTATGGATGAAATGGAATT
       K  A  F  L  H  W  Y  T  G  E  G  M  D  E  M  E  F  408
```

```
5501  TACGGAAGCAGAAAGTAACATGAATGACTTGGTTTCTGAGTATCAACAAT
        T  E  A  E  S  N  M  N  D  L  V  S  E  Y  Q  Q    424
5551  ATCAGgttgttattttcctaatttttctactaatgtaaagtttgccggtt
      Y  Q                                                426
5601  aaattagttacatcacaaaatatttataaaaagctctttcaacaaattct
5651  gaataaaacaaagtaatcttatacaaatattgaaattttaattatagatg
5701  aaactaataattgcttatcattgtgccttttatttatctctcattgcag
5751  GATGCAAGTGCTGATGATGAATTAAATGAAACAATCGAACAAGCGGAAAC
       D  A  S  A  D  D  E  L  N  E  T  I  E  Q  A  E  T  443

5801  AGAATAAaactattgttgcactgcaatttttcttttaagcattgtcaaca
        E  *                                              444
5851  tcctatcagcttgtcaattttttttttgttttctttcaaagaagtttgc
5901  atttatcag
```

Fig. 3.16. Nucleotide sequence of the ß-tubulin gene of *O. gibsoni*. Coding region sequences are shown in upper-case letters; intron and flanking region sequences are shown in lower-case letters. The predicted amino acid sequence is shown below the nucleotide sequence for the coding regions. The limits of both introns GT../..AG) are underlined. The number of the first nucleotide of each line is indicated on the left margin, and the last amino acid of each line is indicated on the right margin of the figure..

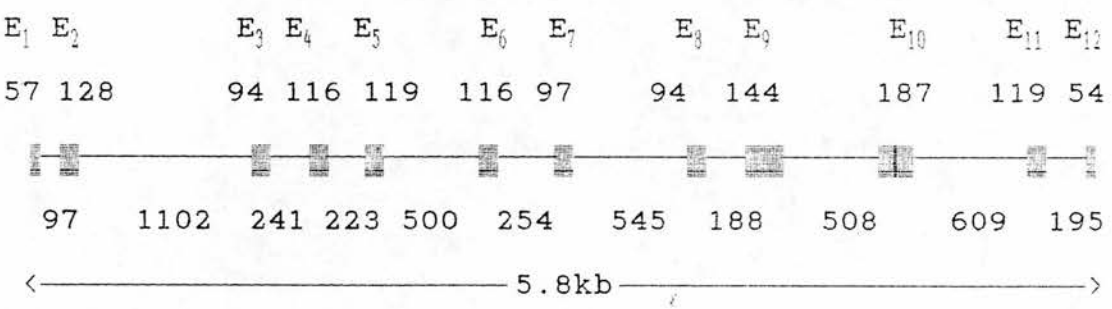


Fig. 3.17. Structure and organisation of ß-tubulin gene from *Onchocerca gibsoni*. The boxed regions represent the DNA coding region for the protein, a total of 12 exons (E1-E12), interspersed with lines representing the 11 introns. The upper numbers indicate the size of the exons, and the lower numbers the size of the introns.

3.11. ANALYSIS OF THE DNA SEQUENCE OF ß-TUBULIN GENE AND THE DEDUCED AMINO ACID SEQUENCE.

The complete nucleotide and derived amino acid sequence of the ß-tubulin gene are shown in Fig. 3.16. The coding region is interrupted by 11 introns, which will be characterized in more detail in another section in this chapter. The coding sequence has 444 codons translating into a protein closely similar to those of other ß-tubulins. The predicted protein has an estimated M_r of 49.95kD.

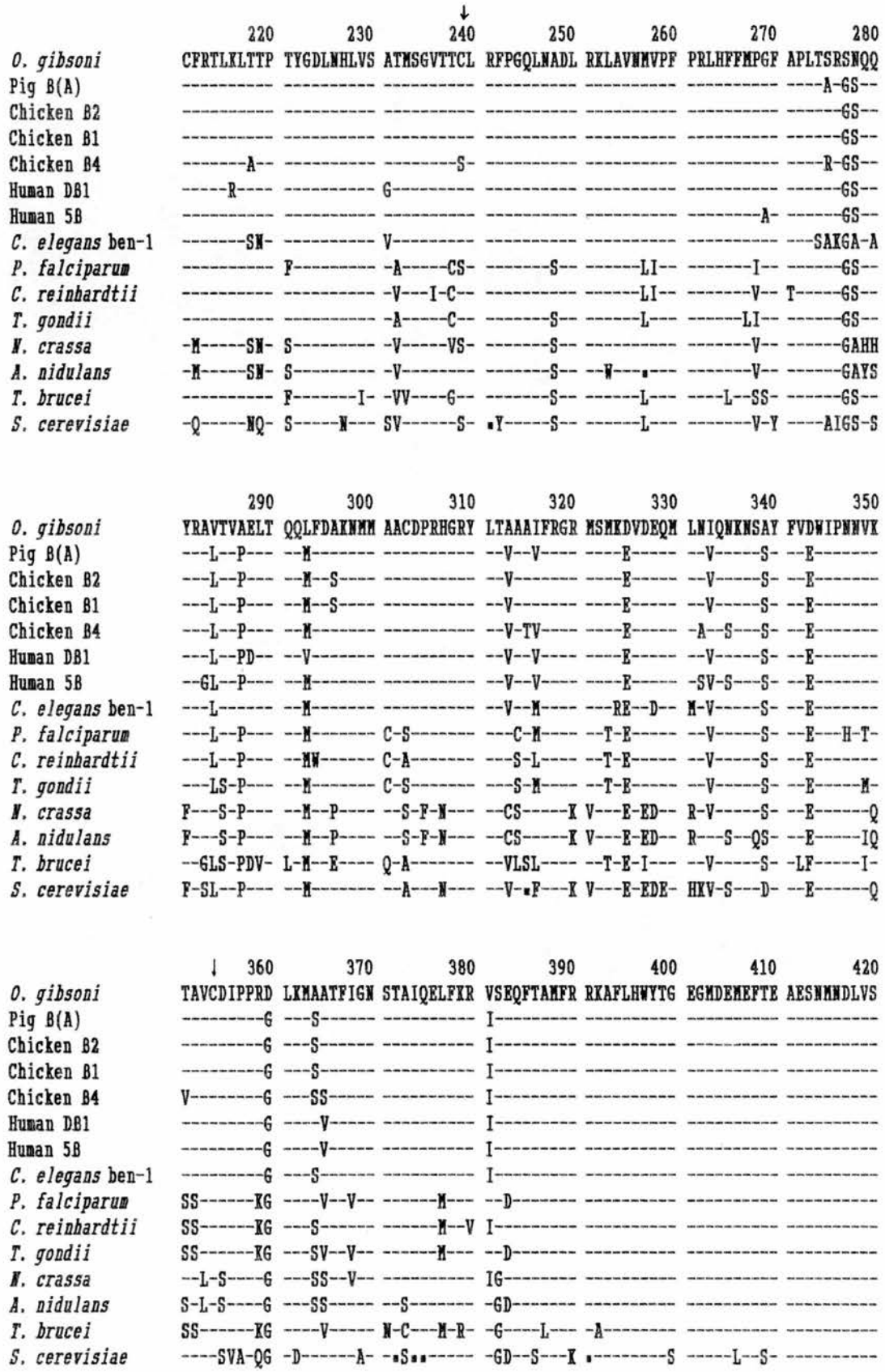
The alignment of the *O. gibsoni* ß-tubulin amino acid sequence with a representative range of sequences from other organisms (parasitic and free-living unicellular organisms, lower and higher metazoans, including human) reveals a number of points of interest (Fig. 3.18). The levels of similarity of the predicted polypeptide to other ß-tubulins are high, ranging from 74.6% (*S. cerevisiae*) to 91.7% (Pig ß(A)) (Fig. 3.18).

Figure 3.18. Comparison of *O. gibsoni* β-Tubulin Sequence with Other β-Tubulin Sequences

```
                        10        20        30        40        50        60        70
O. gibsoni              MREIVHIQAG QCGNQIGSKF WEVISDEHGI DPLGQYHGDS DLQLERINVY YNEVQKKRYV PRAILVDLEP
Pig B(A)                -------W-- W------A-- ---------- --T------- ---------- ---AAGNK-- ----------
Chicken B2              -------W-- W------A-- ---------- --T-S----- ---------- ---ATGNK-- ----------
Chicken B1              -------W-- W------A-- ---------- --T-S----- ---------- ---AAGNK-- ----------
Chicken B4              -------W-- W------A-- ---------- --S-N-V--- -------S-- ---ASSHK-- ----------
Human DB1               -------W-- W------A-- ---------- --T-T----- ----D--S-- ---ATGGK-- ----------
Human 5B                ------LW-- W------A-- ---------- --T-T----- ---------- ---ATGGK-- ---V------
C. elegans ben-1        ------V--- -------A-- ---------- Q-D-T-K-E- ---------- ---ANGGK-- ---V------
P. falciparum           ---------- -------A-- ---------- --S-T-C--- ------VDVF ---ATGG--- -----M----
C. reinhardtii          -------WG- W------A-- ---V------ --T-T----- ---------- F--ATGG--- -----M----
T. gondii               ------V-G- -------A-- ---------- --T-T-C--- --------VF ---ATGG-F- -----M----
N. crassa               ------L-T- -------AA- -QT--G---L -AS-V-N-T- E-----M-V- F--ASGNK-- ---V------
A. nidulans             -----▪L-T- -------AA- -QT--G---L -GS-V-N-T- ------M-V▪ F--ASGNK-- ---V------
T. brucei               -----CVW-- W------A-- ---------- --T-T-Q--- ---------- FD-ATGG--- --SV-I----
S. cerevisiae           ----I--S-- -Y-----AA- --T-CG---L -FN-T---HD -I-K--L-V- F--ASSGKW- --S-N-----

                        80        90        100       110       120       130       140
O. gibsoni              GTNDSVRAGA FGQLFRPDNY VFGQSGAGNN WAKGHYTEGA ELVDSVLDVI RKEAEACDCL QGFQFTHSLG
Pig B(A)                -------S-P ---I-----F ---------- ---------- ---------V ---------- ----L-----
Chicken B2              -------S-P ---I-----F ---------- ---------- ---------V ---S-S---- ----L-----
Chicken B1              -------S-P ---I-----F ---------- ---------- ---------V ---S-S---- ----L-----
Chicken B4              -------S-- --H------F I--------- ---------- ---------V ---C-N---- ----L-----
Human DB1               -------S-P ---I-----F ---------- ---------- ---------V -----S---- ----L-----
Human 5B                -------S-P ---I-----F ---------- ---------- ----A----V -----S---- ----L-----
C. elegans ben-1        -------S-P ---------F ---------- ---------- ----N----V -----G---- ----L-----
P. falciparum           ---------P ---------F ----T----- ---------- --I-A----- -----G---- ----I-----
C. reinhardtii          -------S-P ---I-----F ----T----- ---------- --I------V ---S-S---- ----VC----
T. gondii               ---------P ---------F ----T----- ---------- --I------V -----G---- ----I-----
N. crassa               ----A----P ---------F ---------- ---------- ----Q----V -R---G---- ----I-----
A. nidulans             ----C----P --E------F ---------- ---------- ----N-V--V -R---G---- ---▪I-----
T. brucei               ---------P ---I-----F I--------- ---------- --I------C C-V--S---- ----IC----
S. cerevisiae           W-I-A--NS- I-N------- I----S---V ---------- ------M--- -R---G--S- ----I-----

                        150       160       170       180       190       200       210
O. gibsoni              GGTGSGNGTL LISKIREEYP DRINTTFSVV PSPKVSDTVV EPYNATLSVH QLVENTDETF CIDNEALYDI
Pig B(A)                ---------- ---------- ----N----- ---------- ---------- ---------Y ----------
Chicken B2              ---------- ---------- ----N----M ---------- ---------- ---------Y ----------
Chicken B1              ---------- ---------- ----N----M ---------- ------V--- ---------Y ----------
Chicken B4              ---------- ----V----- ----N----- ---------- --------I- ---------Y ----------
Human DB1               ---------- ---------- ----N----- ---------- ---------- ---------Y ----------
Human 5B                ---------- --------F- ----N----- ---------- ---------- ---------Y ----------
C. elegans ben-1        ---------- ---------- ----SS---- ---------- ---------- ---------- ----------
P. falciparum           ---------- ---------- ----E----F ---------- ---------- -----A--VQ V---------
C. reinhardtii          ---------- ---------- --N-L----- ---------- ---------- -----A--CN VL--------
T. gondii               ---------- ----V----- ----E----F ---------- ---------- -----A--VQ V---------
N. crassa               ----A----- --------F- --N-A-▪--- ---------- ---------- -----S---- ----------
A. nidulans             ----A----- --------F- --N-A----- ---------- ---------- ----HS-▪-- ----------
T. brucei               ------A--- ----L--Q-- ----N---II ---------- ----T----- ----TPN-SN ----------
S. cerevisiae           ---------- -F---K--L- --N-▪----L ----T----- ---------- ----HS---- ----------
```


220 230 240 250 260 270 280
O. gibsoni CFRTLKLTTP TYGDLMHLVS ATMSGVTTCL RFPGQLMADL RKLAVMMVPF PRLHFFMPGF APLTSRSMQQ
Pig B(A)
Chicken B2
Chicken B1
Chicken B4
Human DB1
Human 5B
C. elegans ben-1
P. falciparum
C. reinhardtii
T. gondii
N. crassa
A. nidulans
T. brucei
S. cerevisiae
290 300 310 320 330 340 350
O. gibsoni YRAVTVAELT QQLFDAKMMM AACDPRHGRY LTAAAIFRGR MSMKDVDEQM LMIQMKMSAY FVDWIPMMVK
Pig B(A)
Chicken B2
Chicken B1
Chicken B4
Human DB1
Human 5B
C. elegans ben-1
P. falciparum
C. reinhardtii
T. gondii
N. crassa
A. nidulans
T. brucei
S. cerevisiae
360 370 380 390 400 410 420
O. gibsoni TAVCDIPPRD LXMAATFIGM STAIQELFKR VSEQFTAMFR RKAFLHWYTG EGMDEMEFTE AESMMNDLVS
Pig B(A)
Chicken B2
Chicken B1
Chicken B4
Human DB1
Human 5B
C. elegans ben-1
P. falciparum
C. reinhardtii
T. gondii
N. crassa
A. nidulans
T. brucei
S. cerevisiae

	430	440 444	Total of different bases (in 444 aa)	Percentage of Identity (in 444 aa)
O. gibsoni	EYQQYQDASA	DDELNETIEQ AETE*		
Pig ß(A)	--------T-	-- Q G - F- -EGEEDEA*	37	91.7%
Chicken ß2	--------T-	-- Q G - F- -FGEEDEA*	40	91.0%
Chicken ß1	--------T-	-- Q G - F- -FGEEDEA*	41	90.8%
Chicken ß4	--------T-	EE- G- M Y - DDEEESEQGAE*	42	90.5%
Human DB1	--------T-	EE- - D F G-EAEEEA*	45	89.9%
Human 5B	--------T-	- Q G - F- -EAEEEVA*	45	89.9%
C. elegans ben-1	------E-T-	EED G- LDG -DGDAE*	55	87.6%
P. falciparum	--------T-	EE- G- F- - -EGDVEA*	63	85.8%
C. reinhardtii	----------	EE- G- F-G - -EEA*	65	85.4%
T. gondii	--------T-	EE- G- FD - -EGEMGAEEGA*	65	85.4%
N. crassa	--------GV	-E- - - -Y-EEAPLEGEE*	82	81.6%
A. nidulans	---------I	S-G - - -YAEEEIMEGEE*	85	80.9%
T. brucei	--------TI	EE- G- FD - -EY*	94	78.9%
S. cerevisiae	------E-TV	E-D - - VD -NGDFGAPQNQDE PITENFE*	113	74.6%

Fig. 3.18. Alignment of the predicted amino acid sequence of *O. gibsoni* ß-tubulin (top line) with a representative sample of ß-tubulin from other organisms, together with the percentage identity to the *O. gibsoni* molecule in each case. Only residues different from those in the filarial sequence are shown. Every tenth amino acid is indicated by the cross adjacent to the appropriate number. Black squares mark amino acid positions at which mutational change in the ß-tubulin gene is responsible for a change in benomyl sensitivity in the organism; arrows mark the colchicine binding site. Residue changes are: *S. cerevisiae*; R to H (aa 241; Thomas *et al.*, 1985), R to W (aa 318), T to P (aa 372), I to V (aa 374), Q to R (aa 375), R to T (aa 391; Huffaker *et al.*, 1988); *N. crassa*: F to Y (aa 167; Orbach *et al.*, 1986); *A. nidulans*: A to V (aa 165; B.R. Oakley, personal comunication); *C. elegans*: benzimidazole-sensitive ß-tubulin (Driscoll *et al.*, 1990). Mutations at position 6, 50, 134, 198, 257 causing altered benzimidazole sensitivity in *A. nidulans* (Jung *et al.*, 1989) have not been published in full.

The divergence between the amino acid sequences of the *O. gibsoni* ß-tubulin and *S. cerevisiae* is greater than that of other ß-tubulins sequences in this comparison. The magnitude of the difference between the *O. gibsoni* ß-tubulin and other *Ascomycete* fungi is illustrated by a comparison with *N. crassa* ß-tubulin and *A. nidulans* ß-tubulin. These differ from *O gibsoni* ß-tubulin sequence by 82 and 85 amino acids respectively, whereas *S. cerevisiae* differs by 113 amino acids. The *O. gibsoni* ß-tubulin gene shows the greatest sequence similarity to the ß-tubulin of Pig ß(A). Only 37 amino acids differ between these two genes, corresponding to 91.7% similarity. This similarity to the pig ß-tubulin

molecule exceeds the similarity to the ß-tubulin of a number of organisms, such as the free-living soil nematode *C. elegans* (87.6%), protozoa (*P. falciparum*, 85.8%; *C. reinhardtii* and *T. gondii*, 85.4%; *T. brucei*, 78.9%), and fungi (*N. crassa*, 81.6%; *A. nidulans*, 80.9%; *S. cerevisiae*, 74.6%). Thus, the *O. gibsoni* ß-tubulin gene shows a higher degree of similarity with ß-tubulins from higher eukaryotes, such as those of chicken (ß2, 91%; ß1, 90.8%; ß4, 90.5%), and human (Dß1, and 5ß, 89.9%).

Compared to the amino acid sequences of lower and higher eukaryotes, the *O. gibsoni* sequence has characteristic substitutions at several positions (Table 3.2). The analysis of these substitutions by means of the Venn diagram (Fig. 3.19.) showed that in general the substitutions are conservative in nature. The most common exchanges involve alanine for proline or replacement of one hydrophobic amino acid by another such as the exchange of leucine for isoleucine. The *O. gibsoni* ß-tubulin differs from the other ß-tubulins in the above comparison (Fig. 3.18) in 15 positions: 11 (Gln → Typ), 18 (Ser → Ala), 54 (Val → Ala), 58 (Arg → Lys), 80 (Ala → Pro), 84 (Leu → Ile), 90 (Tyr → Phe), 277 (Ser → Gly), 284 (Val → Leu), 287 (Ala → Pro), 325 (Asp → Glu), 333 (Ile → Val), 339 (Ala → Ser), 360 (Asp → Gly), 381 (Val → Ile).

The proline residues are known to be major determinants of protein conformation, since they occur at a very high frequency in reverse turns. In *O. gibsoni* ß-tubulin however two very conserved proline residues were replaced by alanine at positions 80 and 287. This could contribute to structural differences between host and parasite molecules which might be exploitable by an inhibitor.

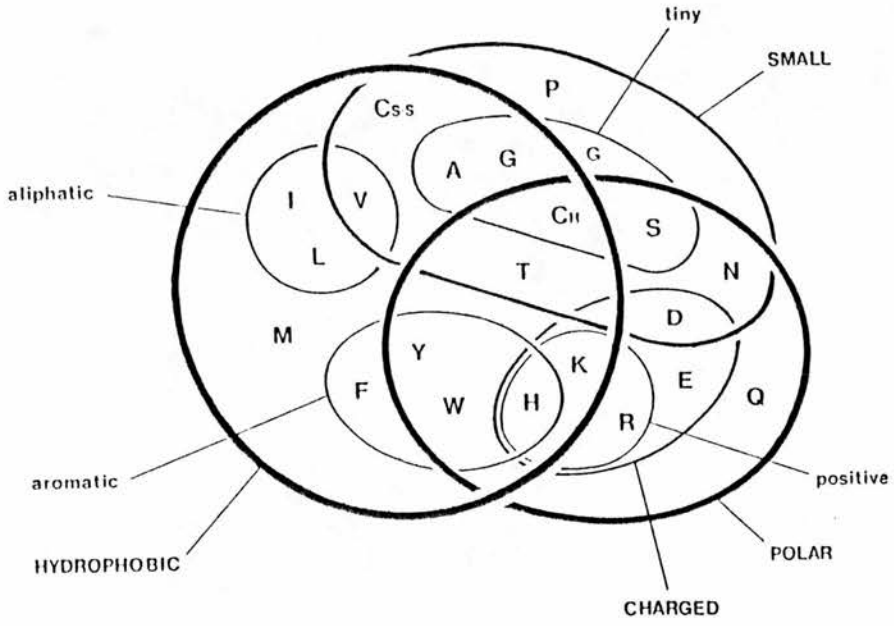


Fig. 3.19. The Venn diagram shows the relationship of the 20 naturally occurring amino acids to a selection of physico-chemical properties which are important in the determination of protein tertiary structure. The diagram is dominated by properties relating to size and hydrophobicity. The amino acids are divided into two major sets, one containing all amino acids which contain a polar group (POLAR) and a set which exhibits a hydrophobic effect (HYDROPHOBIC). A third major set, SMALL, is defined by size and contains the nine smallest amino acids. Within this is an inner set of smaller residues, TINY, which have at most two side chain atoms. The location of Cys is ambigyous and two locations are indicated. Other sets include full-charge (referred to as CHARGED) which contains the subset POSITIVE (negative is defined by implication) and AROMATIC and ALIPHATIC. The latter set is not as general as the name implies and includes only those residues containing a branched aliphatic side chain. Because of its unique backbone properties, proline was excluded from the main body of the diagram.

Table 3.2. Amino Acid Substitutions in *O. gibsoni* ß-Tubulin

O. gibsoni amino acid	*Higher or lower eukaryote* amino acid	*amino acid* position
Gln	Trp, Ser	8
Gln	Trp	11
Ser	Ala	18
Leu	Thr, Ser, Asp, Asn	33
Gln	Ser, Asn, Thr, Val	35
Val	Ala	54
Gln	Thr, Ala, Ser, Asn	55
Lys	Gly, Ser, Asn	56
Lys	Asn, His, Gly	57
Arg	Lys	58
Ala	Ser, Asn	78
Ala*	Pro	80
Leu	Ile	84
Tyr	Phe	90
Ile	Val, Cys	120
Ala	Ser, Asn, Gly	127
Phe	Leu, Val, Ile	135
Thr	Asn, Ser, Leu, Met, Glu, Ala	165
Phe	Tyr, Met, Gln	200
Ser	Gly	277
Asn	Ser, Thr, Ala	278
Val	Leu	284
Ala*	Pro	287
Leu	Met, Val	293
Ala	Val, Cys	313
Ile	Val, Met, Leu, Phe	316
Asp	Glu	325
Ile	Val	333
Ala	Ser	339
Asp	Glu, Phe	343
Asp	Gly	360
Val	Ile	381
Ser	Thr, Gly	429

3.11.1. Clusters of Extreme Conservation in ß-tubulin protein

The sequence conservation among the polypeptides is not, however, randomly distributed throughout the polypeptide, but rather localized into clusters of heterogeneity and clusters of extreme conservation. For example, regions consisting of aa 1-7, 58-77, 91-113, 127-

150, 170- 194, and 391-429 of ß-tubulin show very strong sequence conservation (Fig. 3.18).

The high degree of conservation is not surprising in view of the large number of conserved structural interactions of tubulin. The tubulin dimer has two GTP binding sites (Linse & Mandelkow, 1988), one exchangeable and the other not, a single colchicine binding site (Kilmartin, 1981), two binding sites for vinblastine (Wilson *et al.*, 1975; Wilson & Morse, 1978), and other drug binding sites (Ireland *et al.*, 1979; Laclette *et al.*, 1980; Lacey & Prichard, 1986). In addition, the tubulin structure must be constrained by the requirements for the α-ß dimer interaction (Kirchner & Mandelkow, 1985), for the association between neighbouring dimer units to form protofilaments and microtubules, and for various accessory proteins that may play a regulatory role in assembly.

The most striking example of sequence conservation lies between residues 391 and 429. This region is identical in all currently known ß-tubulins including yeast and is known to be involved in polymerization of tubulins (Serrano *et al.*, 1984). The fact that a third of the residues over this polypeptide stretch chain are charged is consistent with its location at the molecular surface which allows for the interactions involved by polymerization. In this region the sequence of ß-tubulin from *O. gibsoni* is nearly identical to those of the other proteins.

Conserved stretches of amino acids, such as: 1-4 (MREI), involved in translational autoregulation of ß-tubulin mRNA level (Yen *et al.*, 1988), 63-77 (AILVDLEPGTMDSVR), -- region IV -- the binding site of the guanine moiety of GTP (Linse & Mandelkow, 1988), 127-150,

containing a cluster of glycine (GGGTGSG) -- region I -- that forms part of a typical structure between a ß-sheet and an α-helix and resembles a phosphate binding site seen in other nucleotide-binding proteins (Schulz & Schirmer, 1979; Kraus *et al.*, 1981), and 178-181 (TVVE), implicated in the end of another ß-sheet and the ribose binding site (Leberman & Egner, 1984), 391-429, containing residues (EYQQYQDA) involved in polymerisation of tubulins (Serrano *et al.*, 1984), are also found to be very conserved in *O. gibsoni* ß-tubulin (Fig. 3.20).

The conserved residues of Cys at aa position 239 and 354, thought to be involved in the binding of colchicine (Little & Luduena, 1986), are also present in *O. gibsoni* ß-tubulin.

Figure 3.20. Clusters of Extreme Conservation in ß-Tubulin Sequences and their Probable Functions.

```
                    1     7
O. gibsoni          MREIVHI
Pig ß(A)            -------
Chicken ß2          -------
Chicken ß1          -------
Chicken ß4          -------
Human DB1           -------
Human 5B            ------L
C. elegans ben-1    ------V
P. falciparum       -------
C. reinhardtii      -------
T. gondii           ------V
N. crassa           ------L
A. nidulans         -----•L
T. brucei           -----CV
S. cerevisiae       ----I--
```

The ß-tubulin RNAs are selectively targeted as substrates for destabilization through co-translational recognition of the amino-terminal ß-tubulin tetrapeptide, MREI, after its emergence from the ribosome (Yen *et al.*, 1988).

```
                    58                  77
O. gibsoni          YV PRAILVDLEP GTMDSVR
Pig ß(A)            -- ---------- -------
Chicken ß2          -- ---------- -------
Chicken ß1          -- ---------- -------
Chicken ß4          -- ---------- -------
Human DB1           -- ---------- -------
Human 5B            -- ---V------ -------
C. elegans ben-1    -- ---V------ -------
P. falciparum       -- -----M---- -------
C. reinhardtii      -- -----M---- -------
T. gondii           F- -----M---- -------
N. crassa           -- ---V------ ----A--
A. nidulans         -- ---V------ ----C--
T. brucei           -- --SV-I---- -------
S. cerevisiae       W- --S-N----- W-I-A--
```

The binding site of the GUANINE moiety of GTP on ß-tubulin was located within the peptide consisting of residues 63-77, AILVDLEPGTMDSVR, called region IV (also in Fig. 1.12, section 1.5.4) (Linse & Mandelkow, 1988).

```
                    91                  113
O. gibsoni          VFGQSGAGNN WAKGHYTEGA ELV
Pig ß(A)            ---------- ---------- ---
Chicken ß2          ---------- ---------- ---
Chicken ß1          ---------- ---------- ---
Chicken ß4          I--------- ---------- ---
Human DB1           ---------- ---------- ---
Human 5B            ---------- ---------- ---
C. elegans ben-1    ---------- ---------- ---
P. falciparum       ----T----- ---------- --I
C. reinhardtii      ----T----- ---------- --I
T. gondii           ----T----- ---------- --I
N. crassa           ---------- ---------- ---
A. nidulans         ---------- ---------- ---
T. brucei           I--------- ---------- --I
S. cerevisiae       I----S---V ---------- ---
```

This is the most conserved region in the ß-tubulin sequences known thus far. Its function is however unkown.

```
                    127                 150
O. gibsoni          CDCL QGFQFTHSLG GGTGSGMGTL
Pig B(A)            ---- ----L----- ----------
Chicken B2          ---- ----L----- ----------
Chicken B1          ---- ----L----- ----------
Chicken B4          ---- ----L----- ----------
Human DB1           ---- ----L----- ----------
Human 5B            ---- ----L----- ----------
C. elegans ben-1    ---- ----L----- ----------
P. falciparum       ---- ----I----- ----------
C. reinhardtii      ---- ----VC---- ----------
T. gondii           ---- ----I----- ----------
N. crassa           ---- ----I----- ----A-----
A. nidulans         ---- ---•I----- ----A-----
T. brucei           ---- ----IC---- ------A---
S. cerevisiae       --S- ----I----- ----------
```

A glycine-rich stretch, GGGTGSG, called region I, probably represents the turn between a ß-strand and an α-helix as part of a nucleotide--binding motif which is involved in the binding of the PHOSPHATES (Schulz & Schirmer, 1979, Kraus *et al.*, 1981).

```
                    171                 194
O. gibsoni          PSPKVSDTVV EPYNATLSVH QLVE
Pig B(A)            ---------- ---------- ----
Chicken B2          ---------- ---------- ----
Chicken B1          ---------- ------V--- ----
Chicken B4          ---------- --------I- ----
Human DB1           ---------- ---------- ----
Human 5B            ---------- ---------- ----
C. elegans ben-1    ---------- ---------- ----
P. falciparum       ---------- ---------- ----
C. reinhardtii      ---------- ---------- ----
T. gondii           ---------- ---------- ----
N. crassa           ---------- ---------- ----
A. nidulans         ---------- ---------- ----
T. brucei           ---------- ----T----- ----
S. cerevisiae       ----T----- ---------- ----
```

Region II (TVVE residues) is thought to represent the end of another ß-strand that could contribute to the binding of the RIBOSE (Leberman & Egner, 1984).

```
                    391                                 429
O. gibsoni          RKAFLHWYTG EGMDEMEFTE AESNMNDLVS EYQQYQDA
Pig B(A)            ---------- ---------- ---------- --------
Chicken B2          ---------- ---------- ---------- --------
Chicken B1          ---------- ---------- ---------- --------
Chicken B4          ---------- ---------- ---------- --------
Human DB1           ---------- ---------- ---------- --------
Human 5B            ---------- ---------- ---------- --------
C. elegans ben-1    ---------- ---------- --------I- ------E-
P. falciparum       ---------- ---------- ---------- --------
C. reinhardtii      ---------- ---------- ---------- --------
T. gondii           ---------- ---------- ---------- --------
N. crassa           ---------- ---------- ---------- --------
A. nidulans         ---------- ---------- ---------- --------
T. brucei           -A-------- ---------- ---------- --------
S. cerevisiae       •--------S -----L--S- ---------- ------E-
```

EYQQYQDA residues (+ C-t residues DEQG from human ß-tubulin) are directly involved in the interaction with MAP-2 and tau (Maccioni *et al.*, 1988).

3.11.2. Cluster of Heterogeneity in ß-Tubulin Sequences

The most pronounced variable cluster is confined to the extreme C-terminal residues beyond amino acid position 430 (Fig. 3.18). This region is heterogeneous in length as well as in sequence. The C-terminal region of *O. gibsoni* also shows this heterogeneity, since it is somewhat shorter than most of the other ß-tubulin sequences, and contains a particular sequence of residues.

However, comparison of several C-terminal residues shows that in some cases the sequences within the clusters of amino acid divergence have been highly conserved during evolution (Table 3.3). For example, the C-terminal variable regions of chicken ß1, chicken ß2, and pig are essentially identical (Table 3.3). Furthermore, the ß-tubulins from mouse ß3 and human ß2 are also identical to each other in this region. A similar situation exists for an additional pair of mouse ß5 and human M40 ß-tubulin subunits. The conservation of variable region sequences in functionally related tubulins of different species suggests that sequence divergence in these regions is not selectively neutral, but rather that specific sequences have been evolutionarily maintained by positive selective pressure. Thus, these variable domains may define specific ß-tubulin isotypes, since most of the differences between otherwise fairly conserved tubulins are found in this region. The C-terminal region of ß-tubulin has been particularly useful for establishing relationships between various tubulins. But for *O. gibsoni* ß-tubulin no sequence similarity in this region was found among all the ß-tubulin genes sequences known so far.

Table 3.3. Carboxy-terminal Regions of ß-Tubulins

ß-Isotypes	C-Terminus	Class
1 *O. gibsoni*	DASADDE---LNETI----EQAETE	
2 Human M40	DATAEEEED-FG-------EEAEEE-A	1
3 Human ß4	DATAEEEGEMY----EDDEEESESQGPK	3
4 Human 5ß	DATA-EQGE-F-------EEEAEEEVA	4a
5 Human ß2	DATAEEEGE-F-------EEEAEEEVA	4b
6 Pig	DATADEQGE-F-------EEEGEEDEA	2
7 Rat RBT.3	EEEED-FG-------EEAEEE-A	1
8 Rat	DATADEQGE-F------EEEEG-EDEA	2
9 Rat RBT.2	EEGE-F-------EEEAEEEVA	4a
10 Mouse ß5	DATAEEEED-FG-------EEAEEE-A	1
11 Mouse ß3	DATAEEEGE-F-------EEEAEEEVA	4b
12 Mouse ß4	DATA-EEGE-F-------EEEAEEEVA	4a
13 Mouse ß2	DATADEQGE-F------EEEEG-EDEA	2
14 Mouse ß1	DVRAGLEDSEEDA------EEAE-VEAEDKDH	
15 Chick ß1	DATADEQGE-F-------EEEGEEDEA	2
16 Chick ß2	DATADEQGE-F-------EEEGEEDEA	2
17 Chick ß4	DATAEEEGEMY----EDDEEESE-QGAK	3
18 Chick ß3	DATAEEEGE-F-------EEEGEEEAE	
19 Chick ß5	DATANDGFEAF----EDDEEEINE	
20 Chick ß6	DATADVE-E-Y--------EEAEASPEKET	
21 Drosophila ß1	DAEADEDAE-F-----EEEQEAEVDEN	
22 Drosophila ß2	EATADEEGE-F----DEDEEGGGDE	
23 Drosophila ß3	EATADD--E-FDPE-VDQEEVEGDCI	
24 Sea Urchin ß3	DATAEEEGE-F------DEEEGEDEEAA	
25 Sea Urchin ß1	DATAEEEGE-F------DEEEG-DEEAA	
26 *P. myxamoeba*	DATIDDEEGG-EEEEGGAEE	
27 *S. cerevisiae*	EATVEDDEEV-DENGDFGAPQNQDE	
28 *S. pombe*	EAGIDEGDEDY-----EIEEEKEPLDY	
29 *N. crassa*	DAGVDEEEEEY------EEEAPLEGEE	
30 *C. reinhardtii*	DASAEEEGE-F---------EGEEEEA	
31 *T. brucei*	DATIEEEGE-F------DEEEQY	
32 *P. falciparum*	DATAEEEGE-F------EEEEGD-VEA	
33 *B. pahangi* Bpa7	DATADEEGD-L------QEGESEYIE-QEE	

References: 1, The present work; 2-3, Lee *et al.* (1984); 4, Lewis *et al.* (1985); 5, Sullivan & Cleveland (1986); 6, Krauhs *et al.* (1981); 7, Ginzburg *et al.* (1986); 8-9, Lemischka & Sharp (1982); 10-14, Wang *et al.* (1986); 15, Sullivan *et al.* (1986); 16, 18-19, Sullivan & Cleveland (1986); 17, Sullivan & Cleveland (1984); 20, Murphy *et al.* (1986); 21-23, Rudolph *et al.* (1987); 24-25, Alexandraki & Ruderman (1983); 26, Singhofer-Wowra *et al.* (1986a, b); 27, Hiraoka *et al.* (1984); 28, Neff *et al.* (1983); 29, Orbach *et al.*, 1986); 30, Youngblom *et al.* (1984); 31, Kimmel *et al.* (1985); 32, Delves *et al.*, (1989); 33, Helm *et al.* (1989).

In addition, most of the ß-tubulin sequences end with alanine, and contain a phenylalanine, or sometimes tyrosine that is flanked by acidic amino acids (Table 3.3), which is conspicuous among the more conserved amino acids of the ß-tubulin C-terminus. Nevertheless, in *O. gibsoni* the final alanine is absent and phenylalanine was replaced by leucine, and in the filarial worm *B. pahangi* phenylalanine was also replaced by leucine (Table 3.3).

A second and a third markedly divergent cluster in the ß-tubulin sequence are found between residues 33 and 59 and between residues 277 and 383 respectively (Fig. 3.18). In these regions, the divergence observed in *O. gibsoni* ß-tubulin seems to be more similar to higher organisms than for example to the free-living nematode *C. elegans*.

3.11.3. G+C Content, Codon Usage, and Amino Acid Composition

The *O. gibsoni* ß-tubulin is 60% AT rich in the coding region, whilst in the noncoding region the A-T richness is elevated to 80%. This bias is reflected in the selection of codons utilised by the parasite with 74.5% of the codons possessing either A or T in the third position (Table 3.4). Four codons are not used at all, with a further six being used only once. These six codons predominantly have either a G or C base in the third position (Table 3.4.).

As to amino acids, alanine and leucine are the most abundant in the protein. Glycine, glutamic acid, aspartic acid, valine, and threonine are also present at a markedly elevated level. However, cysteine and tryptophan are present at significantly reduced levels (Table 3.4.).

Table 3.4. Frequency of Codon Usage in *O. gibsoni* ß-Tubulin Gene.

Am. Acid	Codon	Number[P]	Percentage[Q]	Total
Gly	GGG	0	0.0	
Gly	GGA	10	33.3	
Gly	GGT	17	56.7	
Gly	GGC	3	10.0	30
Glu	GAG	4	12.9	
Glu	GAA	27	87.1	31
Asp	GAT	26	89.6	
Asp	GAC	3	10.3	29
Val	GTG	4	13.3	
Val	GTA	11	36.7	
Val	GTT	13	43.3	
Val	GTC	2	6.7	30
Ala	GCG	2	5.9	
Ala	GCA	12	35.3	
Ala	GCT	16	47.1	
Ala	GCC	4	11.7	34
Arg	AGG	1	4.3	
Arg	AGA	4	17.4	
Arg	CGG	1	4.3	
Arg	CGA	6	26.1	
Arg	CGT	10	43.6	
Arg	CGC	1	4.3	23
Ser	AGT	8	33.3	
Ser	AGC	1	4.2	
Ser	TCG	6	25.0	
Ser	TCA	2	8.3	
Ser	TCT	4	16.7	
Ser	TCC	3	12.5	24
Lys	AAG	4	25.0	
Lys	AAA	12	75.0	16
Asn	AAT	20	87.0	
Asn	AAC	3	13.0	23
Met	ATG	17	100.0	17
Ile	ATA	3	15.0	
Ile	ATT	12	60.0	
Ile	ATC	5	25.0	20
Thr	ACG	7	23.3	
Thr	ACA	13	43.4	
Thr	ACT	7	23.3	

Table 3.4. Continued

Am. Acid	Codon	Number[P]	Percentage[Q]	Total
Thr	ACC	3	10.0	30
Trp	TGG	4	100.0	4
Cys	TGT	3	37.5	
Cys	TGC	5	62.5	8
Tyr	TAT	12	75.0	
Tyr	TAC	4	25.0	16
Leu	TTG	13	38.2	
Leu	TTA	10	29.5	
Leu	CTG	5	14.7	
Leu	CTA	0	0.0	
Leu	CTT	4	11.8	
Leu	CTC	2	5.8	34
Phe	TTT	16	69.6	
Phe	TTC	7	30.4	23
Gln	CAG	5	20.8	
Gln	CAA	19	79.2	24
His	CAT	9	90.0	
His	CAC	1	10.0	10
Pro	CCG	7	38.9	
Pro	CCA	8	44.4	
Pro	CCT	1	5.6	
Pro	CCC	2	11.1	18
End	TGA	0	0.0	
End	TAG	0	0.0	
End	TAA	1	100.0	1

[P] The total usage of a particular codon in the complete coding sequence.
[Q] The frequency at which a particular codon is used expressed as a percentage of the total codons for that amino acid.

3.11.4. Analysis of the Intervening Sequence

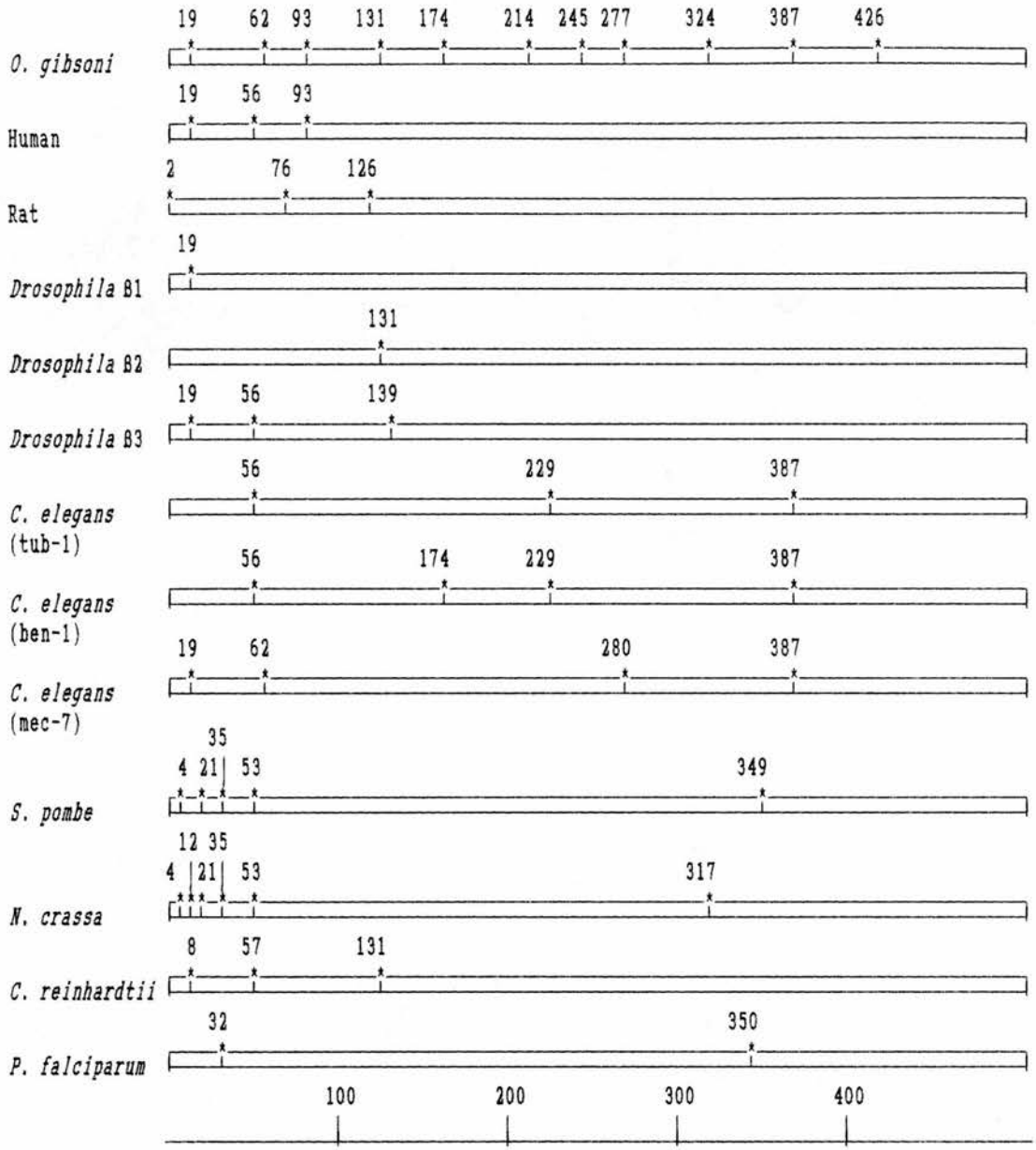
3.11.4.1. Number, Position and length of Introns

The presence of introns in the *O. gibsoni* ß-tubulin gene was inferred by comparison of the deduced amino acid sequence with other ß-tubulin genes (Fig. 3.18). This revealed that the coding region of the ß-tubulin gene from *O. gibsoni* is interrupted by 11 introns fairly evenly spaced throughout the gene (Table 3.5).

Table 3.5. Number, positon and Length of the introns in ß-Tubulin Gene from *O. gibsoni*

Intron Number	Position in the aa sequence	Length (in bp)
1	19	93
2	62	1101
3	93	240
4	131	222
5	174	499
6	214	253
7	245	544
8	277	188
9	324	507
10	387	608
11	426	194

This finding is in marked contrast to the structure of ß-tubulin genes observed in other organisms which have only a few introns. Furthermore most of these introns are clustered within the first 5'quarter of the genes (Fig. 3.21).



No introns: *T. brucei rhodesiense, S. cerevisiae.*

Fig. 3.21. Position of introns within the coding region of tubulin genes. Asterisks indicate introns occurring after or within the codons for the given amino acid. Positions in each case may be plotted against the scale at the bottom. References: Human (Lee *et al.*, 1984); Rat (Lemischka & Sharp, 198); *Drosophila* B1, B2 and B3 (Rudolph *et al.*, 1987); *C. elegans* (Culloti, personal communication); *S. pombe* (Neff *et al.*, 1983); *N. crassa* (Orbach *et al.*, 1986); *C. reinhardtii* (Youngblom *et al.*, 1984); *P. falciparum* (Delves *et al.*, 1989); *T. brucei rhod.* (Kimmel *et al.*, 1985); *S. cerevisiae* (Hiraoka *et al.*, 1984).

A comparison of the position of introns in the ß-tubulin genes from different organisms suggests that some introns appear to be present in common positions within the genes, whilst the position of other introns appear to be unique to a particular gene (Fig. 3.21). The first intron in *O. gibsoni* gene occurs after the 19th codon, a position at which the first intron is also observed in human, *Drosophila* ß1 and ß3, and also *C. elegans* mec-7 genes. An intron in close proximity to this position is found in *N. crassa*. The second *O. gibsoni* intron is present after codon 62, and this is the same position of the second intron in *C. elegans* mec-7 genes. In all the other organisms an intron is found in close proximity to this position with the exception of *Drosophila* ß1, ß2, and *P. falciparum*. The third intron in the *O. gibsoni* gene occurs after codon 93, the same position at which the third intron in human is also located. The fourth *O. gibsoni* intron is located after codon 131 and can be also found in the same position in *C. reinhardtii*; introns in close proximity to this position are also observed in rat, and *Drosophila* ß2 and ß3 genes. The fifth intron in *O. gibsoni* gene occurs after codon 174; it is in the same location as an intron in *C. elegans* ben-1. The eighth intron occurs at codon 277 in the *O. gibsoni* gene, a position in close proximity to a *C. elegans* mec-7 intron. The ninth intron of the *O. gibsoni* gene is at position 324, which is very close to a *N. crassa* intron. Intron number 10 from the *O. gibsoni* gene appears to be nematode specific, as it is located in an identical position in all the three *C. elegans* genes considered. This notwithstanding, the sixth, seventh, and eleventh introns have no corresponding counterpart in all of the other genes.

It has been suggested that the functional domains of proteins are encoded by distinct exons and that the sorting and shuffling of exons may be one mechanism of

protein evolution (Gilbert, 1978). The separation of these exons would permit their shuffling about the chromosome and their reassembly to form various specific genes (Gilbert, 1978). These ideas have been supported by a growing wealth of evidence which suggests the existence of genes with mosaic structures. A vivid example indicating that this assemblage of exons has occurred can be found in the gene of the Low Density Lipoprotein (LDL) receptor (Südhof *et al.*, 1985). Each of the functions of the LDL receptor, binding cholesterol, attaching to the cell surface, traversing the cell membrane, for example, depends on a discrete region or domain, and each domain is produced from several exons. Even more strikingly the LDL receptor appears to be a mosaic protein built up of exons shared with different proteins.

There also appears to be a strong concordance between the exons and the proposed functional domains of the protein in the case of the ß-tubulin gene. All the domains described in chapter 2 fall within distinct exons and some of the introns appear to clearly demarcate functional domains (Fig. 3.22). The clearest example is the domain, involved in the polymerisation of tubulin, which begins immediately after the 10th intron and ends five nucleotides before the 11th intron.

As the position of most of the introns in the ß-tubulin genes coincide with each other, it is possible to speculate that a putative ancestral gene existed which had all of these introns plus all of those which appear to be unique to a particular gene. During the course of evolution differential intron loss took place giving rise to the present day structures of the genes (Antoine & Niessing, 1984).

The lengths of introns in higher eukaryotic genes vary over a considerable range, with vertebrate

genes having, on the average, longer introns than do invertebrate or plant genes (Hawkins, 1988). Most introns in the nematode *C. elegans* are very short, usually around 50 nucleotides (nt) (Emmons, 1988; Blumenthal & Thomas, 1988), approximately 20 nt shorter than introns in Drosophila genes (Hawkins, 1988) . The length of the introns in ß-tubulin gene of *O. gibsoni* (Table 3.5) differs considerably from the length of the majority of the introns in lower eukaryotes including yeast, *Drosophila*, and even the free-living nematode *C. elegans*. It will be of interest, as additional genes are sequenced, to see whether *Onchocerca*, like lower eukaryotes, contains short introns or more resembles higher eukaryotes, where introns are frequent and much longer.

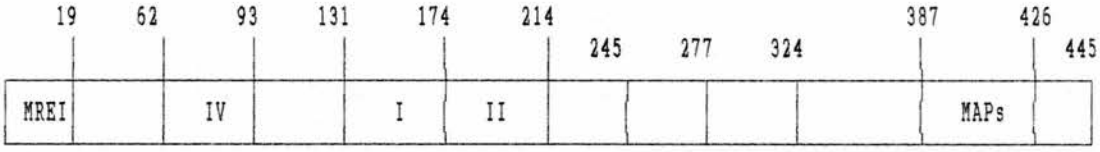


Fig. 3.22. Possible functional domains demarcated by introns in *O. gibsoni* ß-tubulin gene. MREI: amino-terminal tetrapeptide involved in translational autoregulation of ß-tubulin mRNA levels; IV: Region IV implicated in binding the guanine moiety of GTP; I: Region I containing a cluster of glycine that probably forms part of a typical structure between ß-sheet and an α-helix in the phosphate binding site; II: Region II implicated in binding ribose; MAPs: region involved in the polymerisation of ß-tubulin.

3.11.4. Splice Signals

The genomic organisation of *Onchocerca* genes has not been investigated in detail and to date only a few *Onchocerca* genomic clones have been isolated and characterised (section 1.7 in chapter 2). Seven introns from the myosin-like gene and the only intron from the 2 major sperm protein genes have been sequenced.

An analysis of the 5' and 3' splice junction of

these introns was undertaken to further characterize the splice sites (Fig. 3.6, 3.7, and 3.8). Such analysis reveals that the 5' and 3' dinucleotides at the splice donor and acceptor sites conform to the consensus sequence deduced from a wide range of organisms. However the internal nucleotides deviate markedly from this consensus. In this study the exon/intron splice junctions of all 11 introns from the ß-tubulin gene (Table 3.10) add further convincing evidence to support the contention that these junctions do not conform beyond the 5'gt and 3'ag bases of the universal consensus sequences derived from either vertebrates (Table 3.10), plants or yeast (Table 3.9). As there is no unifying consensus sequence observed in *Onchocerca* introns in nucleotides adjacent to the 5' and 3' invariant dinucleotides this suggests that the splice machinery requirements are less stringent at this position (Table 3.9).

An additional sequence thought to be involved in the formation of the lariat intermediate in the splicing mechanism is found in fungal and metazoan introns. This sequence is located within 18-38 nucleotides of the 3' splice site. In yeast the splice signal is TACTAAC but in metazoan this signal is not as rigid, YNYTRAY (Langford & Gallwitz, 1983; Keller & Noon, 1984). The *O. gibsoni* ß-tubulin gene introns do not have any matches with the TACTAAC sequence. Candidates sequences related to the metazoan consensus do occur in all of the introns but the location of these sequences are different from those observed in other organism.

The splicing of introns usually proceeds by cleavage at the 5' splice consensus sequence. The free end of the intron folds in a way such that it forms a lariat structure with the branch consensus box located in the intron near the 3'-end splice consensus. Next, the G residue at the 5'-end of the intron at the 5'-end splice

junction reacts with the 2'-hydroxyl position on the ribose of the proximal 3' A residue of the branch consensus box. As a result, the 3' splice site is cleaved, the two exons ligated together to form a continuous coding sequence and the excised intron is linearised (Sharp, 1985). This fact suggests that although the early steps in the splicing mechanism in *O. volvulus* may be the same as those found in other organisms, the signals involved in the formation of the lariat structure are probably quite different. But this is not too surprising, for these introns are 75% AT rich. Therefore, the splice signal in *O. volvulus* may have diverged from the consensus sequence. However this A-T richness can not be the only contributing factor as the introns of *P. falciparum* genes for example are often 90% A-T rich yet their splice junctions conform completely to the established universal consensus sequences (Khan *et al.*, manuscript in preparation; Simmons *et al.*, 1987).

Table 3.6. The Intron/Exon Boundaries of a ß-Tubulin gene from *O. gibsoni*

Intron Number	5' junction Exon	Intron		3' junction Intron	Exon
1	AG	gtttcaactt	.....	attcttatag	TT
2	AG	gtgaatccaa	.....	caaatttcag	AG
3	CG	gtgaagcttt	.....	atatttccag	GT
4	AA	gtaatatcat	.....	tttcatttag	GG
5	AG	gtatactaat	.....	taattttcag	GT
6	AG	gtatcacttt	.....	aaaattgcag	AA
7	AA	gtttgtttct	.....	aatattttag	TT
8	AA	gtttgttttt	.....	tttttgatag	GT
9	AG	gtttctatct	.....	ctcgtttaag	GA
10	GG	gttaatttta	.....	aatattttag	CG
11	AG	gttgttattt	.....	ctcattgcag	GA
Consensus	**AG:**	**gtttNttt**	**.....**	**tYatttYag:**	**G**

5' splice site		EXON:	intron							
	G	1	8:	11	0	2	1	2	1	0	0
	A	9	3:	0	0	3	4	4	3	3	1
	T	0	0:	0	11	6	6	2	6	5	7
	C	1	0:	0	0	0	0	3	1	3	3
***O. gibsoni* ß-tubulin**		**A**	**G:**	**G**	**T**	**T**	**T**	**N**	**T**	**T**	**T**

3' splice site									intron		:EXON		
	G	0	0	1	0	1	2	0	0	11	:6	3	
	A	5	4	5	1	0	2	1	11	0	:2	3	
	T	6	5	3	10	10	6	5	0	0	:2	5	
	C	0	2	2	0	0	1	4	0	0	:1	0	
	A/T	**Y**	**A**	**T**	**T**	**T**	**Y**	**A**	**G**	**:G**	***N***	***O. gibsoni* ß-tubulin**	

Table 3.7. The Intron/Exon Boundaries of a Myosin-Like Gene from *O. volvulus*

Intron Number	5' junction Exon	Intron	3' junction Intron	Exon
1	AA:	gtattttc.....	.....aatttatag:	GA
2	TT:	gtaggttt.....	.....ttgtttcag:	TA
3	AG:	gtattctt.....	.....tttattcag:	AC
4	AA:	gtaagtta.....	.....atttttcag:	TC
5	TG:	gtcagttg.....	.....aaattaaag:	GA
6	TG:	gtaagtat.....	.....ttttgttag:	GA
7	GA:	gtaagtct.....	.....tcattacag:	AA
Consensus	**R:**	**gtaRgttt.....**	**.....a/tYttttYag:**	**RA**

5' splice site

			EXON:	intron						
G	1	3:	7	0	0	1	5	0	0	1
A	3	3:	0	0	6	4	0	0	1	1
T	3	1:	0	7	0	2	2	6	5	4
C	0	0:	0	0	1	0	0	1	1	1
***O. volvulus* myosin-like**		**R:**	**G**	**T**	**A**	**R**	**G**	**T**	**T**	**T**

3' splice site

								intron		:EXON		
G	0	0	1	0	1	0	0	0	7	:3	0	
A	3	2	2	1	0	3	1	7	0	:2	5	
T	4	4	4	6	6	4	2	0	0	:2	0	
C	0	1	0	0	0	0	4	0	0	:0	2	
	A/T	**Y**	**T**	**T**	**T**	**T**	**Y**	**A**	**G**	**:R**	**A**	***O. volvulus* myosin-like**

Reference: Erondu & Donelson, 1990.

Table 3.8. The Intron/Exon Boundaries of a Major Sperm Protein Gene from *O. volvulus*

Intron Number	5' junction Exon	Intron	3' junction Intron	Exon
1	AC:	gtattatc.....	.....attttcag:	AT
1	AG:	gtattttg.....	.....tgatttcag:	AT
Consensus	**AG/C:**	**gtatta/ttg/c.....**	**.....a/tg/ta/ttttcag:**	**AT**

5' splice site	EXON:	intron							
G	0	1:	2	0	0	0	0	0	1
A	2	0:	0	0	2	0	0	1	0	0
T	0	0:	0	2	0	2	2	1	2	0
C	0	1:	0	0	0	0	0	0	0	1
***O. volvulus* MSP 1&2**	**A**	**G/C:**	**G**	**T**	**A**	**T**	**T**	**A/T**	**T**	**G/C**

3' splice site	intron									:EXON		
G	0	1	0	0	0	0	0	0	2	:0	0	
A	1	0	1	0	0	0	0	2	0	:2	0	
T	1	1	1	2	2	2	0	0	0	:0	2	
C	0	0	0	0	0	0	2	0	0	:0	0	
A/T	**G**	**/T**	**A/T**	**T**	**T**	**T**	**C**	**A**	**G**	**:A**	**T**	***O. volvulus* MSP 1&2**

Reference: Scott *et al.* (1989)

Table 3.9. Comparison of Consensus Sequences of the Splice Sites of *Onchocerca* Genes with Other Organisms

5' splice site	EXON:	intron							
O. gibsoni ß-tubulin	A G:	G	T	T	T	N	T	T	T
O. volvulus myosin-like	- R:	G	T	A	R	G	T	T	T
O. volvulus MSP 1&2	A G/C:	G	T	A	T	T	A/T	T	G/C
Vertebrate	**A G:**	**G**	**T**	**R**	**A**	**G**	**T**		
Plant	**A G:**	**G**	**T**	**A**	**A**	**G**	**T**		
Yeast		**G**	**T**	**A**	**T**	**G**	**T**		

3' splice site						intron			:EXON		
A/T	Y	A	T	T	T	Y	A	G	:G	*N*	*O. gibsoni* ß-tubulin
A/T	Y	T	T	T	T	Y	A	G	:R	A	*O. volvulus* myosin-like
A/T	G/T	A/T	T	T	T	C	A	G	:A	T	*O. volvulus* MSP 1&2
Y	**Y**	**Y**	**Y**	**Y**	**N**	**Y**	**A**	**G**	**:G**		**Vertebrates**
Y	**Y**	**Y**	**Y**	**Y**	**R**	**Y**	**A**	**G**	**:G**		**Plant**
						Y	**A**	**G**			**Yeast**

Table 3.10. Consensus Sequences of the Splice sites of Vertebrates*

5' splice site				EXON:	intron							
%G	23	14	13	77:	100	0	32	12	84	18	30	22
%A	34	35	62	8:	0	0	60	74	9	15	33	22
%T	15	12	13	8:	0	100	5	7	3	50	17	25
%C	29	38	12	8:	0	0	3	7	4	17	21	31
	--	-	**A**	**G:**	**G**	**T**	**R**	**A**	**G**	**T**	--	--

3' splice site														intron:		EXON		
%G	18	13	15	10	10	7	7	10	9	5	5	5	24	0	0	100:	55	27	24
%A	17	11	8	9	7	4	9	8	10	8	4	9	26	2	100	0:	20	21	19
%T	37	44	46	46	56	59	43	49	41	46	42	46	23	19	0	0:	8	32	28
%C	28	33	32	35	27	30	42	33	40	40	49	41	27	78	0	0:	17	20	28
	--	--	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**N**	**C/Y**	**A**	**G:**	**G**	--	--

*A tabulation of the sequences at assigned 5' and 3' splice sites in approximately 400 vertebrate genes in the Gen Bank Data Base. All examples were included where intervening sequences began with a GT dinucleotide and terminated with an AG dinucleotide (from Stuart & Rosenfeld, 1986).

3.12. MOLECULAR NATURE OF BENZIMIDAZOLE (BZ) RESISTANCE

BZ resistance was first described for the mould *Aspergillus* in a series of papers by Oakley, Morris, and their colleagues. These investigators analyzed a class of mutations that confer resistance or supersensitivity to the antimicrotubule drug benomyl, a benzimidazole. Drug resistance was mapped to three loci, one of which (benA) was shown to encode a ß-tubulin polypeptide (Sheir-Neiss *et al.*, 1978; Oakley *et al.*, 1985).

Molecular studies of BZ resistance in other BZ-susceptible eukaryotes, such as *N. crassa (Orbach et al.*, 1986), *S. cerevisiae* (Thomas *et al.*, 1985; Huffaker *et al.*, 1988), and *C. elegans* (Driscoll *et al.*, 1989), also indicate that it is caused by structural changes in the ß-tubulin gene. Fig. 3.18 highlights the position in the ß-tubulin protein in which single amino acid changes generate resistance or super-sensitivity to benomyl in *S. cerevisiae, A. nidulans*, and *N. crassa*. Each of the point mutations in *N. crassa* (Orbach *et al.*, 1986), at amino acid 167 and in *S. cerevisiae* at amino acid 241 (Thomas *et al.*, 1985) and 318 (Huffaker *et al.*, 1988), confer Bz resistance. In *C. elegans* also a number of amino acid substitutions have been identified in a ß-tubulin gene that confers susceptibility and in two genes that confer resistance (Driscoll *et al.*, 1989). It is not known which of these mutations is essential for resistance, as the binding site is not known.

Note that two nearby mutations in different species, one at residue 165 in *A. nidulans* and the other at residue 167 in *N. crassa*, lead to a resistance. If that is a binding site for this type of drug, there may be a benzimidazole which binds more efficiently to *Onchocerca* tubulin than to the host protein, because they differ at position 165 (Thr in the parasites and Asn in the host).

The difficulties in determining the BZ-binding site arise because the mutations that confer BZ resistance are scattered along the ß-tubulin molecule (Fig. 1.12 in chapter 1), along with those that lead to supersensitivity to BZ. This suggests that allosteric changes in the folding of the molecule are involved. The BZ-binding site may be composed of several domains of the ß-tubulin molecule folded together into a specific conformation (Haber *et al.*, 1981; Lacey, 1988; Stark & Wahl, 1984; Kaur *et al.*, 1988; Roos, 1990).

Identifying the binding sites will help to understand the differential effects of BZs on microtubules from different organisms and provide insights into drug resistance. This knowledge should facilitate the design of more specific and potent antimicrotubule agents.

CHAPTER 4.

FUTURE PROSPECTS

4. Future Prospects

A number of chemically synthesised compounds, in particular benzimidazoles, DEC, ivermectin, and isothiocyanates, have been shown to be of clinical promise in the treatment of onchocerciasis. The possible modes of action of these drugs have been described in chapter 1. These drugs often interfere with essential metabolic pathways and neuromuscular function. The primary target of benzimidazoles are tubulins; consequently they interfere with the assembly of microtubules. Since tubulins have been identified as a chemotherapeutically exploitable target, they need to be further investigated. A clearer perception of the mode of action of benzimidazoles awaits a greater knowledge of the detailed structural comparison of parasite tubulins and their genes with their equivalents in the host. This is a necessary step towards the rational design of new antifilarial drugs using molecular principles.

In this study the chromosomal copy of the ß-tubulin gene from *O. gibsoni* has been completely cloned and sequenced. This has revealed the gene to possess a very complex structure compared to the organisation of all the known ß-tubulin genes. The coding region is highly conserved and is interrupted by 11 introns.

It appears certain that both α- and ß-tubulins exist in multiple forms in many, if not all, eukaryotic organisms. In multicellular organisms tubulins have also been shown to be differentially expressed with respect to developmental timing and/or tissue specificity (Villasante *et al.*, 1986; Murphy & Wallis, 1983; Sullivan & Cleveland, 1986). In unicellular organisms different forms of tubulins have been associated with different locations and functions (Russell *et al.*, 1984; Green & Dove, 1984).

Thus, it would be of interest to determine the number of genes coding for each tubulin and to investigate the expression of these genes in the different stages of the filarial worms life cycle. In many prokaryotic and eukaryotic organisms gene regulation appears to be controlled at the level of RNA expression (Ptashne, 1986; Struhl, 1987). Eukaryotic promoters are composed of many *cis*-acting control motifs some of which can form binding sites for transcription factors that enhance transcription. It may be possible to visualise these distinctive promoter elements by a simple comparison of the upstream sequences in the *Onchocerca* ß-tubulin gene which can be determined from the existing N-terminal clone.

Recent investigations have revealed that trypanosomes and nematodes share a novel post-transcriptional process called trans-splicing (Boothroyd and Cross, 1982; Krause & Hirsh, 1987; Donelson & Zeng, 1990). In this process a short spliced leader or mini-exon sequence is joined to a protein coding precursor RNA molecule to create the mature mRNA. In the case of trypanosome this 39 nucleotides spliced leader sequence is joined to all mRNA species and in nematodes a different 22 nucleotide spliced leader sequence is joined to mRNAs encoding cytoskeletal proteins, metabolic enzymes, ribosomal proteins and regulatory proteins. As tubulin is a cytoskeletal protein, it will be of interest to investigate if the spliced leader sequence is joined to the message. This can be determined by cloning and sequencing the cDNA or by primer extension analysis.

The expression of the *O. gibsoni* ß-tubulin gene in a heterologous host, such as *E. coli*, would allow the generation of a polyclonal antiserum. This would provide an invaluable tool to elucidate the tissue specific distribution of the protein. However the multiple introns

within the gene would pose a problem in the expression of the gene as *E.coli* lacks the intron splicing machinery. This can be circumvented by applying the polymerase chain reaction technique to amplify the intronless gene from either messenger RNA or from an aliquot of the cDNA library with the appropriate primers.

A collection of varied molecules that have been defined on the basis of their binding and/or putative interaction with microtubules, such as MAP and tau proteins, may be important in the regulation of microtubule assembly. These microtubule-associated proteins in *Onchocerca* can be isolated and characterised by affinity chromatography using the expressed protein as a ligand. Solubilised extracts of *O. gibsoni* can be passed through a column containing the recombinant ß-tubulin coupled to sepharose and the bound proteins eluted and analyzed by polyacrylamide gel electrophoresis. Bands of interest can be excised from the gel and used to generate a polyclonal antiserum and the association with the ß-tubulin verified by electronmicroscopy. The genes for proteins of interest can be isolated by immunoscreening *Onchocerca* expression libraries and then characterised.

Understanding the molecular biology of *Onchocerca* would be advanced by the application of recombinant DNA technology together with protein chemistry. Further research into this intriguing parasite has great medical potential and promises to be biologically fascinating.

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